Inhibitory Effects of Securinine and Related Compounds on Toxoplasma gondii and Mechanistic Insights into Transcript-Specific Translational Repression in the Bradyzoite Developmental Stage

Michael James Holmes
University of Windsor

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Inhibitory effects of securinine and related compounds on *Toxoplasma gondii* and mechanistic insights into transcript-specific translational repression in the bradyzoite developmental stage

By

Michael Holmes

A Dissertation

Submitted to the Faculty of Graduate Studies
through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy
at the University of Windsor

Windsor, Ontario, Canada

2015

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Inhibitory effects of securinine and related compounds on *Toxoplasma gondii* and mechanistic insights into transcript-specific translational repression in the bradyzoite developmental stage

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January 16, 2015
Declaration of Co-Authorship / Previous Publication

I. Declaration of Co-Authorship

I hereby declare that this dissertation incorporates material that is the result of joint research, as follows:

Chapter 2: This work is the outcome of a joint research undertaken in collaboration with Anna K. Crater and Bhartesh Dhudshia under the supervision of Dr. Sirinart Ananvoranich and Dr. Avinash N. Thadani. Anna K. Crater and Sirinart Ananvoranich contributed to data collection. Bhartesh Dhudshia synthesized the chemicals used in the study under the supervision of Dr. Avinash N. Thadani.

Chapter 3: This work incorporates data generated by Vaunell Itaas under the supervision of Dr. Sirinart Ananvoranich. Vaunell Itaas constructed some of the plasmids used for the deletional analysis of the $LDH1$ 5’UTR and performed some of the related data acquisition.

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ABSTRACT

Toxoplasma gondii is an intracellular protozoan parasite that infects warm blooded animals the world over. In the host, the parasite exists as either rapidly growing tachyzoites or immunologically protected slowly dividing bradyzoites. While tachyzoites are generally cleared by the host’s immune system, bradyzoites persist for life. If the immune system becomes compromised, bradyzoites reconvert to tachyzoites and cause severe illness. Bradyzoites develop in response to stressful stimuli, therefore discovering factors that modulate bradyzoite conversion as well as determining how Toxoplasma’s stress response pathways operate are essential to understanding parasite disease. In this work, the plant alkaloid securinine as well as ten pyrrolidine derivatives were shown to have in vitro anti-Toxoplasma activity in the micromolar range. These compounds act in different capacities, either restricting parasite’s invasion or by reducing the parasite’s rate of replication. One of the hallmarks of the bradyzoite life stage is slowed replication. When tested, only securinine induced bradyzoite formation at comparable levels to treatment with alkaline media, suggesting that it initiates a stressful stimulus.

When in the bradyzoite stage, Toxoplasma induces a global translational repression allowing for the remodeling of the transcriptome. However it remains unclear how certain transcripts are maintained throughout the long term translational repression seen in bradyzoites. Analysis of one such transcript, lactate dehydrogenase 1, showed a 40 nucleotide cis-acting element with its 5’UTR responsible for this activity. The formation of 16 nucleotide regulatory RNA hairpin is necessary for inducing sustained translational repression in bradyzoites, suggesting that it acts as a recognizable feature. Characterization of the transcript reveals that its poly(A) tail does not shorten in bradyzoites, indicating that
it is likely targeted for long term storage instead of degraded under stress. Efforts were made to capture and analyse the protein complement interacting with the $cis$-acting element using RNA aptamer technologies, however they were unfruitful. Using extracellular parasites, it was determined that a rapid induction of stress is insufficient to selectively repress the transcript. This suggests that the reprogramming of the proteome may be necessary for the sustained translational repression of lactate dehydrogenase 1 in *Toxoplasma* bradyzoites.
ACKNOWLEDGEMENTS

I would like to begin by acknowledging my supervisor, Dr. Sirinart Ananvoranich for her mentorship and support. You have taught me so much and I will carry these lessons along with me for life. Also, thank you for giving me a great opportunity to work on such an exciting project, for which I am very grateful. I would also like to thank my committee members, Dr. Boffa, Dr. Crosby, and Dr. Vacratsis, who I could always count on for useful advice.

I would like to extend my thanks to my lab colleagues. Anna, thank you for your useful advice, for being a great sounding board to bounce ideas off, and for helping me keep perspective. Emad, thank you for your kindness and help. To the rest – Ahmed, Biju, Dema, Farzana, Ionut, Urszula, and the many undergrads: thank you all for sharing your time and lives with me. I would also like to acknowledge the technical assistance of Vaso Globarevic and Sirin Chaker. To others in the department: thank you for your conversations, I am grateful for what I have learned from each of you. I would like to extend my best wishes to all.

Most importantly, I would like to recognize my friends and family, particularly Jocelyn, Lucie, and Hanna, who have been with me through it all. Thank you for sharing in my triumphs and setbacks, listening even though what I was saying didn’t always make sense, and for consistently encouraging me throughout.
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<tbody>
<tr>
<td>4E-BP</td>
<td>eIF4E-binding protein</td>
</tr>
<tr>
<td>AGO</td>
<td>Argonaute</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>AP2</td>
<td>Apetala 2 transcription factor</td>
</tr>
<tr>
<td>ARE</td>
<td>AU-rich element</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAG1</td>
<td>Bradyzoite antigen 1</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CP</td>
<td>MS2 phage coat protein</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
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<td>DHFR</td>
<td>Dihydrofolate reductase</td>
</tr>
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<td>DHPS</td>
<td>Dihydropteroate synthase</td>
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<td>DIG</td>
<td>Digoxigenin</td>
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<td>Dimethyl sulfoxide</td>
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<td>DNA</td>
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<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
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<tr>
<td>DOZI</td>
<td>Development of zygote inhibited</td>
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<td>Acronym</td>
<td>Description</td>
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<td>-------------</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>eIF</td>
<td>Eukaryotic initiation factor</td>
</tr>
<tr>
<td>EJC</td>
<td>Exon junction complex</td>
</tr>
<tr>
<td>ENO</td>
<td>Enolase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
</tr>
<tr>
<td>FF</td>
<td>Firefly</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>GABA</td>
<td>$\gamma$ aminobutyric acid</td>
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<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GRA</td>
<td>Dense granule protein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HDV</td>
<td>Hepatitis delta virus</td>
</tr>
<tr>
<td>HFF</td>
<td>Human foreskin fibroblast</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>Hp</td>
<td>RNA hairpin</td>
</tr>
<tr>
<td>HXGPRT</td>
<td>Hypoxanthine-xanthine-guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon γ</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosomal entry site</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>logP</td>
<td>Logarithm of partition coefficient</td>
</tr>
<tr>
<td>MIC</td>
<td>Microneme protein</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro RNA</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mRNP</td>
<td>Messenger ribonucleic particle</td>
</tr>
<tr>
<td>NAD+</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>nCBC</td>
<td>Nuclear cap-binding complex</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<td>NRE</td>
<td>Nanos response element</td>
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<td>Open reading frame</td>
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<td>PABP</td>
<td>Poly(A)-binding protein</td>
</tr>
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<td>P-body</td>
<td>Processing body</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>Full Form</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein disulfide isomerase</td>
</tr>
<tr>
<td>PDTC</td>
<td>Pyrrolidine dithiocarbamate</td>
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<tr>
<td>PTGR</td>
<td>Post-transcriptional gene regulation</td>
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<tr>
<td>PUF</td>
<td>Pumilio and FBF protein</td>
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<td>RNA-binding protein</td>
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<td>RFI</td>
<td>Relative fluorescent intensity</td>
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<td>Relative fluorescent unit</td>
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<td>Rhopty neck protein</td>
</tr>
<tr>
<td>ROP</td>
<td>Rhopty protein</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
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<td>Surface antigen</td>
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<td>SAGE</td>
<td>Serial analysis of gene expression</td>
</tr>
<tr>
<td>SBP</td>
<td>Streptavidin-binding peptide</td>
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<tr>
<td>SG</td>
<td>Stress granule</td>
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<tr>
<td>TD_{50}</td>
<td>50% toxic dose</td>
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<tr>
<td>TgDRE</td>
<td><em>Toxoplasma gondii</em> DNA repair enzyme</td>
</tr>
<tr>
<td>TgIF2K</td>
<td><em>Toxoplasma gondii</em> eIF2 kinase</td>
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<tr>
<td>TI</td>
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<td>Target of rapamycin</td>
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<td>Tubulin</td>
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CHAPTER I – General introduction

1.1 Discovery of *Toxoplasma gondii*

*Toxoplasma gondii* is a single-celled protozoan parasite of the phylum Apicomplexa. It is the only species within the genus *Toxoplasma*. *T. gondii* was first discovered independently in an African rodent in Tunisia and in a rabbit in Brazil (Nicolle and Manceaux, 1908; Splendore, 1908). It derives its name from the Greek “toξo” meaning bow-like and the gundi, the animal from which it was first identified (Nicolle and Manceaux, 1909). Since its discovery, *T. gondii* has been found to be a wide-spread parasite capable of infecting virtually any nucleated cell in warm-blooded animals including birds and mammals, earning it the title of the world’s most successful parasite (Tenter *et al.*, 2000; Turner *et al.*, 2013).

1.2 *Toxoplasma* life cycle

*Toxoplasma gondii* is an intracellular parasite that spends most phases of its life cycle inside the cells of its host. This life cycle can be subdivided into the sexual and asexual phases (Figure 1.1). In the sexual life cycle, the parasite differentiates through a progression of cell types that allow for genetic crossing in its definitive host. In the asexual life cycle, the parasite alternates between two stages and reproduces through a clonal division.
Sexual reproduction occurs in the epithelium of the feline’s small intestine. Cats shed oocysts into the environment which are then taken up by warm blooded animals. This causes an acute infection by tachyzoites which differentiate into bradyzoites upon induction of the immune response. Bradyzoites can be ingested through the consumption of infected tissues. Adapted from Black and Boothroyd, 2000.

Figure 1.1 Sexual and asexual life cycles of *Toxoplasma gondii*. 

Sexual reproduction occurs in the epithelium of the feline’s small intestine. Cats shed oocysts into the environment which are then taken up by warm blooded animals. This causes an acute infection by tachyzoites which differentiate into bradyzoites upon induction of the immune response. Bradyzoites can be ingested through the consumption of infected tissues. Adapted from Black and Boothroyd, 2000.
1.2.1 Sexual life cycle

Members of the family Felidae act as the definitive host of *Toxoplasma gondii*. Parasites are first taken up by the cat by ingesting either tissue cysts from an infected animal through predation or sporulated oocysts from the environment (Dubey *et al.*, 1998). Once either of these two forms encounters the feline’s stomach, the cyst wall is dissolved and infectious parasites are released which then infect the epithelial lining of the small intestine (Dubey *et al.*, 1998). Differentiation of the parasite into male and female gametocytes proceeds and these combine to form oocysts (Dubey *et al.*, 1998). Oocysts are then released from the cat through its feces where sporulation occurs in the environment (Dubey *et al.*, 1998). The length of this process is complete in about 1 to 5 days depending on ambient temperature (Dubey *et al.*, 1998). This results in the formation of four sporozoites contained within the sporulated oocyst (Dubey *et al.*, 1998). Sporulated oocysts can survive for years in the environment (Torrey and Yolken, 2013). If they happen to be ingested by a feline, another round of sexual reproduction can occur, thus completing the cycle (Dubey *et al.*, 1998).

1.2.2 Asexual life cycle

Ingestion of tissue cysts or sporulated oocysts from the environment can also lead to the commencement of asexual reproduction in warm blooded animals (Dubey *et al.*, 1998). Any host other than the cat is termed an intermediate host although asexual reproduction also occurs in the feline definitive host (Dubey *et al.*, 1998). Upon reaching the stomach, gastric juices serve to digest the cyst wall and release infectious parasites (Dubey *et al.*, 1998). These invade the host’s tissues where they differentiate into rapidly growing tachyzoites during an acute infection cycle (Dubey *et al.*, 1998). During this phase of acute
infection, tachyzoites can spread throughout the host as well as be vertically transmitted to a pregnant host’s offspring (Montoya and Liesenfeld, 2004). Infection by *Toxoplasma* causes activation of the host’s immune system. In response to signals generated from the immune response, tachyzoites differentiate into slow growing bradyzoites which are immunologically protected (Skariah *et al.*, 2010). Bradyzoites are encased in a cyst wall and persist for the life of the host in a chronic infection (Dubey *et al.*, 1998). Bradyzoites can re-enter the *Toxoplasma* life cycle if an infected animal containing tissue cysts is eaten through carnivorism (Dubey *et al.*, 1998).

1.2.3 Distribution, prevalence and transmission in animals

It is estimated that at any given time, approximately 1% of domestic cats are shedding oocysts into the environment at a rate of up to 55 million each a day per animal (Torrey and Yolken, 2013). It has been estimated that on average, there may be as many as 9 to 434 oocysts per square foot of residential area in the United States of America shed by domestic and feral cats alone (Torrey and Yolken, 2013). This value is assumed to be much higher in areas where cats selectively defecate such as sand boxes and gardens (Torrey and Yolken, 2013). Importantly, studies have shown that a single oocyst is capable of causing infection in animals (Dubey *et al.*, 1998).

*Toxoplasma* is transmitted to animals is through the consumption of sporulated oocysts or infected meat. This most often occurs by eating directly off contaminated soil, eating small insects that carry oocysts, drinking contaminated water, or carnivorism (Dubey and Jones, 2008). They can also become infected by ingesting contaminated milk (Dubey
and Jones, 2008; Tenter *et al.*, 2000). Infection among small rodents is particularly important as they are direct prey for cats.

*Toxoplasma* has been found to chronically infect animals on every continent including Antarctica (Jensen *et al.*, 2012; Dubey and Jones, 2008). *Toxoplasma* infections are not limited to wild animals as the parasite has also been seen in companion animals and other animals held in captivity such as farm and zoo animals (Dubey and Jones, 2008). The infection of livestock is particularly of concern to human health because the ingestion of contaminated undercooked meat or unpasteurized milk can pass the parasite along (Montoya and Liesenfeld, 2004; Dubey and Jones, 2008; Tenter *et al.*, 2000). Pigs, sheep, goats, and chickens are more likely to be infected than cattle and raising these animals in a free range environment increases the risk of infection because they are not protected from oocysts in the environment (Dubey and Jones, 2008).

1.2.4 Distribution, prevalence and transmission among humans

*Toxoplasma gondii* is one of the most wide-spread human parasites with infections noted on every continent. Around the world it is estimated that approximately one third of people are infected with *Toxoplasma* and nearly 200 000 newborns are infected annually (Montoya and Liesenfeld, 2004; Torgerson and Mastroiacovo, 2013). However, infection rates can vary greatly from country to country, even between those that are geographically close to one another. For example, seroprevalence in European countries vary from 7% in the United Kingdom to 67% in Yugoslavia (Lafferty, 2006). In Canada and the United States of America, studies have estimated that between 12-25% of people are seropositive (Lafferty, 2006; Shuhaiber *et al.*, 2003; Kruszon-Moran and McQuillan, 2005). Risks of
infection include cat ownership and raw meat consumption (Jones et al., 2009). Sanitary conditions have also been shown to be important for controlling Toxoplasma infection. For example in 1995, the world’s most serious Toxoplasma outbreak was recorded in Victoria, British Columbia and was attributed to oocyst contamination of the water supply (Mullens, 1996). While Toxoplasma infection is usually acquired orally, other methods of transmission are also possible. These include receiving an infected organ during transplantation or congenital transmission from pregnant mother to fetus (Montoya and Liesenfeld, 2004).

Multiple Toxoplasma genotypes have been found to infect people across the globe. Phylogenetic studies have revealed that most Toxoplasma infections in North Americans and Europeans are caused by three main clonal lineages commonly known as types I, II and III (Howe and Sibley, 1995). Many other Toxoplasma genotypes exist as well. For instance, isolates from South America appear to be much more genetically diverse (Khan et al., 2006). The high prevalence of types I, II, and III is likely due to their highly successful asexual clonal expansion amongst the population (Khan et al., 2006). There is also a correlation between Toxoplasma type and severity of infection. Infections with type II and III strains are less virulent and more common than infection with type I strains (Khan et al., 2006).

1.3 Toxoplasmosis

Infection with T. gondii can cause the disease toxoplasmosis. Infection can be divided into acute, latent, and recurrent phases. The acute phase of infection is caused by rapidly growing tachyzoites and is generally considered to have mild to moderate
symptoms depending on a variety of parasite and host-related factors. The latent phase of infection is associated with the presence of bradyzoites that persist for the life of host. Symptoms are not easily discernible and historically, chronic infection has been considered to be asymptomatic in humans. Immunosuppression followed by the subsequent conversion of bradyzoites into tachyzoites is responsible for recurrent toxoplasmosis. This phase has the most severe effects in adults because the infection is already established throughout the host and the immune system cannot combat the infection (Montoya and Liesenfeld, 2004).

1.3.1 Acute toxoplasmosis

Acute toxoplasmosis occurs during the initial infection of the host with the parasite. In immunocompetent hosts initial infection with T. gondii is generally benign, however mild flu-like symptoms and / or swollen lymph nodes can occur (Montoya and Liesenfeld, 2004). Typically, tachyzoites, the causative agents of acute toxoplasmosis, are cleared by the immunocompetent host’s immune system (Montoya and Liesenfeld, 2004). Therefore, except in rare cases, these symptoms are usually quickly resolved and leave no lasting effects (Montoya and Liesenfeld, 2004). Factors affecting the severity of symptoms include parasite strain and virulence, the number of parasites taken in during initial acquisition, as well as the sex, age, and immunological status of the host (Montoya and Liesenfeld, 2004).

Although a rare occurrence in immunocompetent people, severe cases of acute T. gondii infection can cause serious disease to the eye. Ocular toxoplasmosis can cause temporary cloudy eyesight when reproducing tachyzoites obstruct the path of light in the
eye as well as permanent partial blindness when they destroy localized areas of the retina (Mullens, 1996). While the cloudy vision can clear up once drugs are used to control the initial infection, any destruction of retinal tissue results in permanent eyesight impairment (Montoya and Liesenfeld, 2004).

If a woman becomes acutely infected for the first time during pregnancy, the parasite can be vertically transmitted to the fetus through the placenta, causing congenital toxoplasmosis. Generally, the earlier in gestation that transmission occurs, the more severe effects congenital toxoplasmosis can have on fetal development (Montoya and Liesenfeld, 2004). The most severe symptoms include problems associated with eyesight such as blindness and many debilitating effects on the brain including enlargement of the skull, atrophy of the brain, the presence of brain lesions, and mental retardation (Montoya and Liesenfeld, 2004). In the most severe circumstances, congenital toxoplasmosis can cause spontaneous abortion or stillbirth, with this being a leading cause of stillbirth and abortive births in sheep (Montoya and Liesenfeld, 2004; Innes et al., 2009).

In contrast to healthy individuals, previously unexposed patients who receive an organ or hematopoietic stem cell transplant from a seropositive donor can suffer severe consequences (Tenter et al., 2000). These include neurological problems and, most severely, a multi organ response which can lead to sepsis, a mostly fatal outcome (Vaughan and Wenzel, 2013).

1.3.2 Latent toxoplasmosis

Latent toxoplasmosis is the term associated the persistent infection of a host with Toxoplasma bradyzoites (Montoya and Liesenfeld, 2004). Once this occurs, infection is
incurable and lasts for life. Virtually any nucleated cell can be infected by the parasite and bradyzoites have been found in many different tissues, including skeletal and cardiac muscle, as well as eye and brain tissues (Montoya and Liesenfeld, 2004). Latent toxoplasmosis has been traditionally considered asymptomatic and because of this, infection rates are under-diagnosed in the population (Montoya and Liesenfeld, 2004; Tenter et al., 2000).

Interestingly, reports have emerged suggesting that latent toxoplasmosis can give rise to long-term behavioural and phenotypic changes, thereby challenging the assumption of asymptomatic infection (reviewed by Flegr, 2013). For example, people who test seropositive for *Toxoplasma* have decreased reaction times in standardized testing and display a higher rate of work-related or traffic accidents than the average population (Flegr, 2013). Furthermore, personality and psychological testing has revealed that infected individuals are more likely to characterize themselves as risk takers and are more prone to psychological disorders including depression, schizophrenia, and suicidal behaviour (Flegr, 2013). Whilst latent toxoplasmosis appears to have nuanced effects on human behaviour, the results can be striking in other animals. For example, chronically infected rats see an abolishment of their innate avoidance of preditorial scents (Berdoy et al., 2000). Instead, they display an attraction to the scent of feline urine, effectively increasing their predation by the parasite’s definitive host in the wild and thus completing the parasite’s lifecycle (House et al., 2011).

These characteristics and diseases may be associated with alterations in hormone and neurotransmitter levels that occur upon long term *Toxoplasma* infection. For example,
increased testosterone levels are seen in chronically infected men, and increases in \(\gamma\)-aminobutyric acid (GABA) and dopamine are seen in rodents (Hodková et al., 2007; Fuks et al., 2012; Stibbs, 1985). Notably, these chemicals are linked to the psychiatric issues described above and \textit{T. gondii} possesses the ability to directly increase the levels of these neurotransmitters as it encodes the complete pathway for converting glutamate to GABA as well as two tyrosine hydroxylases that produce the dopamine precursor L-dopa (MacRae et al., 2012; Gaskell et al., 2009). By altering these chemical levels, the parasite could be thought to subtly increase the mortality rate of those infected, potentially increasing \textit{Toxoplasma}'s ability to come in contact with a new host.

1.3.3 Recurrent toxoplasmosis

Although recent research is illuminating the ways in which chronic toxoplasmosis affects human health, by far the most common and serious pathologies of toxoplasmosis are seen when encysted bradyzoites reconvert to tachyzoites in the host. Referred to as recurrent toxoplasmosis, this process can affect any previously infected individual if they become immunocompromised. The immune status of the host can become compromised due to illnesses or pharmacological interventions, the most common of which include the taking of immunosuppressant drugs following organ transplantation, undergoing chemotherapy as a treatment for various cancers, or the development of acquired immune deficiency syndrome (AIDS) due to HIV infection (Montoya and Liesenfeld, 2004; Tenter et al., 2000).

Because recurrent toxoplasmosis relies on the reactivation of dormant bradyzoites previously disseminated throughout the body, drastic pathologies can emerge following
their conversion into tachyzoites. Common symptoms include ocular complications such as retinitis and toxoplasmic encephalitis (Tenter et al., 2000). Encephalitis occurs when reactivated tachyzoites reproduce and lyse infected neurons causing brain lesions and necrotic foci in the host (Montoya and Liesenfeld, 2004). This damage to the central nervous system can cause speech abnormalities, motor deficits, seizures, changes in mood, psychosis, and is potentially fatal if left untreated (Montoya and Liesenfeld, 2004). Toxoplasmic encephalitis occurs in up to 40% of AIDS patients worldwide and, at the turn of the millennium, complications arising from Toxoplasma infection accounted for approximately 10-30% of the fatal outcomes in seropositive HIV positive individuals (Tenter et al., 2000). Other issues that can arise from the reactivation this opportunistic pathogen are the development of skin lesions; toxoplasmic pneumonia; the swelling of organs including, but not limited to, the heart, liver, and the gastrointestinal tract; and, a disseminated toxoplasmosis leading to sepsis (Lowichik and Ruff, 1995; Tenter et al., 2000).

1.4 Prevention and treatment

Currently, there exists only one Toxoplasma vaccine, a live attenuated parasite strain that is defective in cyst formation (Kur et al., 2009). However, it is only approved for veterinary use in sheep, has a limited shelf life, is prohibitively expensive, and has the potential to revert to a more virulent phenotype (Kur et al., 2009; Liu et al., 2012). Although many attempts have been made, there are no approved vaccines for use in humans and therefore efforts to prevent human infection rely on education, public health initiatives, and careful hygienic practices (Kur et al., 2009; Montoya and Liesenfeld, 2004; Verma and Khanna, 2013).
1.4.1 Approved drug regimens

Toxoplasmosis is usually considered asymptomatic in immune-competent individuals and therefore treatment of *Toxoplasma* infection is limited only to those showing signs of acute infection, newly infected pregnant mothers, or preventative treatment for seropositive individuals at high risk of recurrent toxoplasmosis (Montoya and Liesenfeld, 2004). Anti-toxoplasmosis drug regimens consist exclusively of the administration of multi-drug cocktails (Montoya and Liesenfeld, 2004). The most commonly used is the combination of sulfadiazine and pyrimethamine; alternatively, sulfamethoxazole and trimethoprim may also be used (Montoya and Liesenfeld, 2004). These drugs inhibit the dihydropteroate synthase (DHPS) activity of the bifunctional hydroxymethyl dihydropterin pyrophosphokinase-DHPS enzyme and the dihydrofolate reductase (DHFR) activity of the bifunctional enzyme DHFR-thymidylate synthase, respectively (Aspinall *et al.*, 2002). Together, these drug combinations act synergistically to inhibit *de novo* synthesis of folinic acid in *Toxoplasma* (Aspinall *et al.*, 2002; Meneceur *et al.*, 2008). While the DHPS molecular target of sulfonamides is absent in humans, a DHFR orthologue does exist and therefore, to minimize toxicity to the host, folinic acid is always given in conjunction with pyrimethamine or trimethoprim (Warhurst, 1986; Montoya and Liesenfeld, 2004). Notably, while all animals rely on dietary folates for nucleotide synthesis, *Toxoplasma* is unable to use preformed folates for its metabolic needs, making folinic acid supplementation an effective way to minimize host toxicity (Warhurst, 1986; Aspinall *et al.*, 2002; Montoya and Liesenfeld, 2004).

Although supplementation with folinic acid is standard practise, the use of pyrimethamine in animals has the potential to cause neural tube defects and other issues in
the early phases of fetal development (Montoya and Liesenfeld, 2004; Peters et al., 2007). Within the first two trimesters, the macrolide spiramycin is alternatively used because it achieves high concentrations in the placenta, thereby reducing the potential for fetal transmission (Montoya and Liesenfeld, 2004; Halonen and Weiss, 2013). Another common treatment issue arises in individuals who are intolerant or allergic to sulfonamides. When this occurs, either the protein synthesis inhibitor clindamycin or the cytochrome b inhibitor atovaquone is given in conjunction with pyrimethamine and folinic acid (Menecue et al., 2008; Montoya and Liesenfeld, 2004; Halonen and Weiss, 2013).

Although the currently used drug regimens are generally effective, concerns have arisen regarding the spread of drug insensitivity and resistance. For example, reports have documented the resistance of clinical isolates to sulfonamides from animal and human samples collected in the United Kingdom and France (Aspinall et al., 2002; Menecue et al., 2008). Furthermore, a large variation in susceptibility to pyrimethamine as well as atovaquone has also been demonstrated between clinical isolates (Menecue et al., 2008). While outright resistance towards pyrimethamine has not yet been observed in *Toxoplasma gondii* isolates, this phenomenon is widely spread in *Plasmodium falciparum*, the causative agent of malaria and an apicomplexan parasite (Fohl and Roos, 2003). Two mutations within the *DHFR-TS* gene are required to induce resistance in *P. falciparum*, and sequencing from multiple *T. gondii* clinical isolates shows that *DHFR-TS* is already polymorphic, suggesting potential for the development of pyrimethamine resistance also exists in *Toxoplasma* (Fohl and Roos, 2003; Aspinall et al., 2002; Menecue et al., 2008; toxodb.org v.12.0).
1.4.2 Novel drug discovery

Since currently used drug regimens have severe side effects and have variable efficacies, the discovery of alternative compounds displaying anti-Toxoplasma activity acting against different molecular targets is desirable. Some of the efforts take advantage of the differences between the biology of Toxoplasma and the human host. While these have yet to be translated to a clinical setting, many compounds show high efficacies in vitro or in a mouse model of infection. For example, the herbicide fluridone specifically inhibits the synthesis of the plant hormone abscisic acid by the apicoplast, a parasite-specific organelle of red algal origins (Nagamune et al., 2008; van Dooren and Striepen, 2013). Furthermore, it has been shown to decrease parasite burden in a mouse model and is well tolerated by mammalian cells since, while the production of this hormone is essential for the completion of the parasite’s lifecycle, the pathway is absent in mammalian and avian hosts (Nagamune et al., 2008).

Some drug discovery efforts have taken advantage of the similarities between the apicomplexan parasites Plasmodium falciparum and Toxoplasma gondii. For example the currently used anti-malarial drug artemisinin has shown effectiveness in vitro and in mouse models at inhibiting Toxoplasma growth (Hencken et al., 2010; Schultz et al., 2014). Artemisinin, the active compound in the traditional Chinese herb qinghao, is particularly interesting because its synthetic derivatives are currently the only compounds that are effective at clearing Toxoplasma bradyzoites (Hencken et al., 2010; Schultz et al., 2014). Many more large scale drug screening efforts have been directed towards P. falciparum in part because malaria causes many more fatalities, thus increasing the interest in the development of novel chemotherapeutics. Importantly, these efforts have yielded a library
of approximately twenty thousand compounds that show anti-malarial activity in vitro, 400 of which are freely available (Spangenberg et al., 2013). Screening of these compounds discovered several novel agents with different chemical backbones that display high selectivity against Toxoplasma, suggesting that building upon the work of malaria drug research may eventually yield novel anti-Toxoplasma agents (Boyom et al., 2014).

Another common vein of drug discovery rests upon the repurposing of drugs currently used on humans for non-related diseases since these are generally already known to be well tolerated and therefore may have fewer regulatory hurdles to overcome. Of particular interest is the activity of drugs acting on the central nervous system, since they are known to cross the blood-brain barrier and because the majority of complications from recurrent toxoplasmosis occur in the brain. For example, one study has shown that the antipsychotic haloperidol, which acts in opposition to dopamine, and the mood stabilizer valproic acid, which increases GABA activity and reuptake, inhibit Toxoplasma growth in vitro at concentrations below their bio-availabilities in vivo (Jones-Brando et al., 2003; Kornhuber et al., 1999). While noting the ability of the parasite to synthesize these neurotransmitters (MacRae et al., 2012; Gaskell et al., 2009), these examples may suggest that altering the availability of these neurotransmitters may modulate Toxoplasma growth.

1.5 Lytic cycle

The most serious outcomes of toxoplasmosis are etiologically caused by the destruction of host cells which is itself caused by tachyzoites undergoing multiple replicative cycles. In an immunocompromised host, tachyzoites are free to rapidly divide and invade new cells and do so through a repeating process called the lytic cycle (Black
and Boothroyd, 2000). The lytic cycle can be subdivided into three major parts: the attachment and invasion of an uninfected cell by an extracellular parasite, followed by multiple rounds of intracellular growth and replication of tachyzoites, and completed by the lysis of the host cell with subsequent parasite egress. The newly freed tachyzoites then migrate towards new host cells and the cycle begins anew (illustrated in Figure 1.2).
Extracellular parasites first attach and invade an uninfected host cell. The intracellular parasite undergoes multiple replicative rounds. Once the host cell’s size limit is reached, the tachyzoites escape from the lysed host and the extracellular parasites migrate towards and invade a new host cell. Adapted from Gubbels, 2014.
1.5.1 Attachment and invasion

*T. gondii* is an obligate intracellular parasite and as such, when it finds itself in the extracellular environment, it must attach to the host cell’s membrane and subsequently invade it in order to survive. The initial attachment of the host by the parasite is mediated through the recognition of specific host cell factors, namely host cell surface glycoproteins (Black and Boothroyd, 2000; Robinson *et al.*, 2004; Friedrich *et al.*, 2010). One of the main mediators of attachment is the family of surface antigens, SAGs. The most prominent member, SAG1, is a marker of the tachyzoite stage and has been shown to interact specifically with glucosamine, found on the cell surface (Robinson *et al.*, 2004). The SAG family members are not the only host cell-binding mediators as SAG1 knockout mutants as well as bradyzoites, which do not express SAG1, still attach and invade host cells (Robinson *et al.*, 2004).

More recently, it has been shown that sialic acid plays a major role in the maintenance of the parasite-host interaction. After initial contact is formed with the host cell, the parasite reorients its apical end towards the host cell and secretes the contents of its microneme, an apical secretory organelle which contains the MIC family of adhesins (Soldati-Favre, 2008). The MIC1-4-6 complex and, in an independent complex, MIC13 are anchored to the parasite’s membrane and are responsible for binding sialic acid, one of the most abundant oligosaccharides that are incorporated onto cell surface glycoproteins and glycolipids (Blumenschein *et al.*, 2007; Friedrich *et al.*, 2010). Interestingly, the widespread usage of sialylated conjugates on the cell surface throughout the animal kingdom and in various tissues may be a reason why *Toxoplasma* has such a large tropism (Schauer, 2004). For example, in addition to invading any nucleated cell in any warm
blooded animal, *T. gondii* also successfully invades host cells in which the parasite cannot survive like those of mosquitoes and fish (Buckley, 1973; Omata *et al.*, 2005).

Immediately after the microneme ejects MIC proteins into the parasite’s membrane, another apical secretory organelle, the rhoptry, injects its content of rhoptry neck proteins (RON)s into the membrane of the host cell (Soldati-Favre, 2008). The microneme-derived and parasite-anchored AMA1 interacts with the host cell-anchored RON2, forming a stable link between parasite and host (Lamarque *et al.*, 2011; Tyler and Boothroyd, 2011). The RON2-4-5-8 complex serves to anchor the parasite to the host cell and, together with the AMA1 protein, serve to act as the moving junction. Interestingly, this demonstrates that *Toxoplasma* provides its own cell surface receptor in RON2 and is another indication as to why the parasite can invade a multitude of cell types (Besteiro *et al.*, 2011). Contrary to other intracellular pathogens where the host cell internalizes them, *T. gondii* actively invades its host. The cytoplasmic portion of AMA1, as well that of another adhesin MIC2, interact with the parasite’s aldolase (Jewett and Sibley, 2003; Sheiner *et al.*, 2010). In apicomplexan parasites, in addition to serving a metabolic role, aldolase also serves as the bridge between adhesins and the parasite’s actin cytoskeleton (Jewett and Sibley, 2003).

Through an active process dependent on *Toxoplasma*’s actin-myosin motor system (Jewett and Sibley, 2003), the parasite causes an invagination of the host’s membrane and progressively passes further into the host until it is completely internalized within the parasitophorous vacuolar membrane. This membrane delimits the nonfusogenic parasitophorous vacuole and is composed of host cell lipids and a mix of parasite and selected host cell membrane proteins (Sinai, 2008). *Toxoplasma* secretes proteins from its
rhoptries (ROP)s and its dense granules (GRA)s that integrate within the parasitophorous vacuole to control its fate within the host cell. By reorganising the host’s intermediate filament and microtubular networks, the parasitophorous vacuole migrates towards the nucleus where it stably associates with the endoplasmic reticulum through the action of GRA3 and with mitochondria through a mechanism that may or may not involve the ROP2 family (Laliberté and Carruthers, 2008; Kim et al., 2008; Sinai and Joiner, 2001; Pernas and Boothroyd, 2010).

1.5.2 Growth and replication

As an intracellular parasite, Toxoplasma must coopt the normal functioning of its host cell for its own benefit, converting it from potentially hostile environment to one that promotes the parasite’s growth and replication. The parasite accomplishes this through a variety of mechanisms. For example, a decrease in host cyclin B1 causes it to arrest at the G2/M interface while both the extrinsic and intrinsic apoptosis pathways are inhibited due to increased expression of a variety of anti-apoptotic proteins (Brunet et al., 2008; Carmen and Sinai, 2007). These are important because, as an intracellular parasite, T. gondii would not be able to survive in a cell undergoing apoptosis and would undoubtedly encounter difficulties within an actively dividing host cell. To accomplish these feats, Toxoplasma drastically reprograms the host cell at the transcriptional level by modulating a number of transcription factors upon infection. For example, the polymorphic kinases ROP16 and ROP18, as well as the dense granule proteins GRA2 and GRA15 have been shown to modulate the activities of NF-κB and the STAT family of transcription factors in a strain specific manner (Du et al., 2014; Butcher et al., 2011; Jensen et al., 2013). Alleles of these genes from virulent type I parasites tend to activate STATs and inhibit NF-κB while type
II parasites do the reverse (Du et al., 2014; Butcher et al., 2011; Jensen et al., 2013). Notably, the downstream targets of these transcription factors are varied and include cell cycle and anti-apoptotic genes (Du et al., 2014; Butcher et al., 2011). Importantly, not just protein coding transcripts are modified. Reports have shown that T. gondii specifically induces the expression of certain host miRNAs, some of which are upregulated through the activation of STAT3 and have anti-apoptotic properties (Zeiner et al., 2010; Cai et al., 2013). Taken together, this suggests that Toxoplasma has ideally evolved to remodel the host environment to be conducive for parasite survival and growth.

In addition to modulating the host cell environment, the parasite has developed methods to siphon its nutritional requirements from the host cell. By promoting the degradation of its regulatory partner through an unknown effector, Toxoplasma causes an increase in host hypoxia-inducible-factor-1 (Wiley et al., 2010). Downstream targets of this transcription factor include growth factors and genes that promote increased glucose metabolism and iron uptake (Laliberté and Carruthers, 2008). Toxoplasma is auxotrophic for the amino acids tryptophan, arginine, and serine, purines, cholesterol, certain lipids, and small molecules such as choline and polyamines (Laliberté and Carruthers, 2008). The host’s endo-lysosomal pathway, in conjunction with the modified microtubular network and the activity of GRA7, is used to deliver a constant supply of lipids and cholesterol needed for growth (Coppens et al., 2006). In addition, the parasite is able to gather small molecules such as sugars, amino acids, nucleotides, and ions by diffusion through the porous parasitophorous vacuole membrane (Laliberté and Carruthers, 2008). Together, these processes satisfy not only the parasite’s nutritional needs, but also allow growth and the rapid, efficient replication that is characteristic of tachyzoites.
Parasite virulence is directly tied to the rapidity with which tachyzoites can complete their cell cycle and successfully replicate (Radke et al., 2001). While all *Toxoplasma* strains spend approximately 60% of the cell cycle in G1 and 30% in S phase at a genomic content of 1.8N, virulent type I strains have an increased replication rate, completing the entire cycle in 5-6 hours while slower growing type II tachyzoites take approximately 9 hours (Radke et al., 2001). Since the highest proportion of time is spent in G1, it suggests that the main driving force behind the increase in virulence is the ability for the tachyzoite to grow. Interestingly, as alluded to above, the type I allele of ROP18 correlates with increased virulence (Taylor et al., 2006). When this allele is transformed into a type II strain, the tachyzoites proceed through the cell cycle at an increased rate and become more virulent in mice, thus reinforcing the necessity of remodelling the host environment for optimal growth (Taylor et al., 2006).

*Toxoplasma gondii* displays a streamlined cell cycle compared to most eukaryotic model organisms. For example, the G2 phase is absent and mitotic spindle assembly occurs in late S phase while the parasite only has a genomic content of 1.8N, suggesting a lack of clear delineation between S and M phases (Radke et al., 2001; Gubbels et al., 2008). Furthermore, mitosis occurs in its entirety without the dissolution of the nuclear membrane and with only limited chromosomal condensation (Gubbels et al., 2008). Parasite duplication occurs through a process of internal budding, known as endodyogeny. This produces two immature daughter cells, each delineated by the inner membrane complex, within the mother. The inner membrane complex is a membrane and cytoskeletal network that serves as a scaffold for the recruitment of the nucleus and a complete organellar complement (Ouologuem and Roos, 2014). Shortly after receiving these, the inner
membrane complex pinches closed and the daughters emerge from the mother, leaving behind a residual body whose materials are recycled within the parasitophorous vacuole (Gubbels et al., 2008). The entire process from the commencement of mitosis until the completion of cytokinesis occurs within a matter of 35-40 minutes (Radke et al., 2001).

1.5.3 Parasite egress

In all cases, the most serious consequences of Toxoplasma gondii infection are caused by the destruction of infected cells due to the escape of tachyzoites. Several signals can cause egress and produce viable extracellular parasites. These can be extrinsic factors, such as mechanical disruption of the host cell due to physical strain or from an immune response (Blackman and Carruthers, 2013), or through parasite-driven factors. After repeated rounds of multiplication, the parasitophorous vacuole fills with parasites. The plant hormone abscisic acid is produced by the parasite apicoplast, serving as a quorum sensing molecule to signal the release of intracellular calcium stores (Nagamune et al., 2008; McCoy et al., 2012). The release of calcium activates the calcium dependent protein kinase 3 which induces microneme secretion (McCoy et al., 2012). The micronemes secrete a perforin-like protein that breaks the parasitophorous vacuole membrane (Kafsack et al., 2009). The lysis of the parasitophorous vacuole increases the concentration of intracellular calcium in the host, activating the host protease calpain 1 which in turn degrades the host’s cytoskeleton, facilitating parasite egress (Chandramohanadas et al., 2009; Blackman and Carruthers, 2013). The parasites then liberate themselves from the weakened host cell and in total, this process takes between one and ten minutes (Caldas et al., 2007). Once extracellular, they are free to attach, invade and colonize a new host cell to complete the lytic cycle.
1.6 Bradyzoites

Bradyzoites are the most important developmental phase of the parasite asexual lifecycle. Without this stage, depending on parasite virulence and dosage, a tachyzoite infection would either be rapidly cleared by the immune system or would illicit an overwhelming inflammatory response in the host, ultimately leading to a rapid death. Instead, bradyzoites provide an alternate path, serving to act as a non-immunogenic state where the establishment of an incurable chronic infection for the life of host occurs. Furthermore, they reportedly alter host behaviour in ways that increase host mortality, thereby increasing the odds that they will be ingested by a predator or scavenger and transmit the infection to a new host (House et al., 2011; Flegr, 2013). Bradyzoites have developed a variety of adaptations that aid them in persisting long term within the host cell. One of these includes modifying the parasitophorous vacuole into an elastic, glycoprotein-rich, protective cyst wall whose integrity is crucial for tissue cyst maintenance in vivo (Zhang et al., 2001; Tomita et al., 2013). Other classic features include a stalling of parasite growth and replication as well as a reduction in their metabolic needs.

1.6.1 Changes to growth and metabolism

The modulation of parasite growth is an important step in the establishment of chronic disease. If uncontrolled growth occurs, the host is unable to stem the infection and ultimately succumbs. This is particularly demonstrated by type I parasites, who grow rapidly, are deficient in bradyzoite development, and are considered much more virulent in vivo than type II parasites (Radke et al., 2001; Soëte et al., 1994; Sibley and Boothroyd, 1992). In contrast, parasites that successfully convert to bradyzoites exit the cell cycle in
the G1 phase and remain non proliferative, existing solely with a 1N genomic content (Radke et al., 2003).

Accompanying this decrease in growth and replication is a fundamental shift in metabolism. Bradyzoites likely lack the ability to complete the tricarboxylic acid cycle and their unique mitochondrion loses membrane potential indicating an absence of oxidative phosphorylation (Denton et al., 1996; Toursel et al., 2000). Furthermore, along with an upregulation of the enzymes needed for gluconeogenesis, bradyzoites are marked by the appearance of cytoplasmic amylopectin storage granules whose presence has been hypothesized to serve as an energy reservoir that allows them to survive the transition from one host to another (Fleige et al., 2008; Coppin et al., 2003). Together, these metabolic characteristics underline the importance of glycolysis in bradyzoites, where glycolytic throughput is much greater in the functionally anaerobic bradyzoites than in tachyzoites (Denton et al., 1996).

Interestingly, most of the enzymes involved in the glycolytic pathway are duplicated in the Toxoplasma genome and at least some of these show differential stage expression, enzymatic properties, and/or cellular functions between one another, suggesting specialized adaptations between tachyzoite and bradyzoite life stages has occurred (Fleige et al., 2007; Manger et al., 1998, Dzierszinski et al., 1999; Dzierszinski et al., 2001; Yang and Parmley, 1997; Dando et al., 2001). For example, the bradyzoite-specific lactate dehydrogenase (LDH) 2, which converts pyruvate to lactate and regenerates the essential coenzyme NAD⁺, is much more suited to the bradyzoite environment than the tachyzoite-specific LDH1 even though they share 71% identity (Yang and Parmley, 1997). LDH2 is
able to offer a much larger throughput of NAD\(^{+}\) regeneration because it is insensitive to pyruvate concentration, whereas LDH1 displays substrate inhibition (Dando et al., 2001). The inverse is true in the case of *Toxoplasma* enolases (ENO)s, where the tachyzoite-specific ENO2 displays a higher specific activity than the bradyzoite ENO1 (Dzierszinski et al., 2001). Intriguingly, the *Toxoplasma* ENOs both display nuclear localization and more recently they have been shown to act as stage-specific transcription factors or transcriptional regulators, underlining that stage-specific adaptations are not just limited to enzymatic functionality (Ferguson et al., 2002; Mouveaux et al., 2014).

1.6.2 Stage-specific transcriptional gene regulation

Differential gene regulation between life stages is an essential feature of any organism that needs to adapt to a changing environment. In its infancy, studies directed toward bradyzoite-specific gene transcriptional upregulation in *Toxoplasma* had only revealed a handful of model genes, such as the heat shock protein bradyzoite antigen 1 (*BAG1*), and the metabolic enzymes *LDH2* and *ENO1* (Bohne et al., 1997; Yang and Parmley, 1997; Dzierszinski et al., 2001). In fact, along with verifying the absence of the tachyzoite-specific LDH1, ENO2, and SAG1 proteins, seeing the protein products of these genes had emerged as canonical markers of successful induction of the bradyzoite stage (Ferguson, 2004). The development and application of microarray and SAGE technologies to the *Toxoplasma* transcriptome revealed many more stage-specifically regulated transcripts (Cleary et al., 2002; Radke et al., 2005). These studies showed that upon bradyzoite induction, a remodeling of the transcriptome occurs within the broadly defined families of surface antigens, metabolic enzymes, cell cycle regulators, and proteins targeted to secretory organelles (Cleary et al., 2002; Radke et al., 2005). However, it was unknown
if bradyzoite-induced genes were upregulated transcriptionally or through post transcriptional mechanisms, such as through a systematic increase in their mRNA half-lives (Cleary et al., 2002; Radke et al., 2005). This question was categorically answered when it was shown that the promoters of canonical bradyzoite-induced genes contained cis-acting elements that were capable of inducing bradyzoite-specific developmental regulation when inserted in the promoters of otherwise constitutively expressed genes (Behnke et al., 2008). Furthermore, these cis-acting elements were able to selectively bind unknown nuclear proteins, suggesting they interacted with unknown transcription factors (Behnke et al., 2008).

Perhaps surprisingly, despite the completion of the Toxoplasma genome project and the known importance of so called “just-in-time” transcriptional regulation to apicomplexan cell biology, initial efforts to identify transcription factors had proved largely unfruitful (Bozdech, 2003; Meissner and Soldati, 2005). It wasn’t until relatively recently, when sequence-based homology searches directed against predicted DNA-binding motifs in the Plasmodium genome were performed that a large number of plant-like AP2 transcription factors were suggested to be present and active in apicomplexan parasites (Balaji et al., 2005). Interestingly, when studied in Toxoplasma, not only were many of the newly identified AP2 transcription factors found to be regulated in a cell cycle dependent manner, yet more were specifically upregulated in bradyzoites (Behnke et al., 2010). Furthermore, these transcription factors were suggested to bind different promoter regulatory cis-acting elements, suggesting that these factors mediate the transcription of genes throughout progression and withdrawal from the cell cycle (Behnke et al., 2010). More recently, it has been shown that different bradyzoite-induced AP2 family members
can selectively induce or repress the transcription of the same subset of canonical bradyzoite transcripts, underlining their importance in stress response and developmental transcriptional regulation (Walker et al., 2013; Radke et al., 2013).

1.6.3 Bradyzoite differentiation in vivo

Conversion to the bradyzoite stage offers a means to the parasite to persist in the host indefinitely, and this ability appears to be strain-specific. While the type I lineage of parasites is more virulent and usually fatal in mouse models, type II parasites cause a much less severe disease and readily persist as bradyzoite-containing cysts (Robben et al., 2004). The difference between these outcomes is primarily mediated through modulation of the host’s inflammatory response, which itself is mediated through the interaction between the parasite and macrophages, sentinel cells that are a key component of the innate immune system. Upon host cell egress, type II tachyzoites infect macrophages where they specifically induce the classical inflammatory activation pathway through the activity of the secreted dense granule protein GRA15 (Jensen et al., 2011; Rosowski et al., 2011). Through an unknown mechanism, GRA15 induces the phosphorylation of the IκB kinase which in turn phosphorylates the NF-κB inhibitory protein IκB, causing its ubiquitination and subsequent degradation (Rosowski et al., 2011). The newly freed NF-κB transcription factor then translocates to the nucleus where it initiates the transcription of pro-inflammatory genes such as the cytokines tumour necrosis factor α and interleukin-12 (Rosowski et al., 2011; Robben et al., 2004). These cytokines promote Th1 cell activation and the production of interferon γ (IFNγ) in a variety of cell types, which in turn promotes NF-κB activation, creating a positive feedback loop that induces a robust inflammatory response (Schroder et al., 2004).
IFNγ has long been known to be a key mediator of bradyzoite differentiation and its presence is essential to survival in a mouse model of type II infection (Jones et al., 1986; Suzuki et al., 1988; Suzuki et al., 1991). Among the many downstream targets with anti-parasitic activities that are upregulated by IFNγ are inducible nitric oxide synthase (iNOS) and indoleamine 2,3 dioxygenase (Schroder et al., 2004). While the latter depletes the intracellular environment of tryptophan, iNOS produces the reactive nitrogen intermediate nitric oxide (NO) from arginine (Schroder et al., 2004). *Toxoplasma* is auxotrophic for both of these amino acids and while their depletion could be cause for cellular stress, it is the production of NO that is essential for long term host survival and mediates bradyzoite differentiation (Laliberté and Carruthers, 2008; Scharton-Kersten et al., 1997; Bohne et al., 1994). Molecular targets of NO include enzymes of the Krebs cycle, such as succinate dehydrogenase, and the essential heme groups of the cytochromes involved in complexes I-IV of the mitochondrial respiratory chain (Dai et al., 2013). Notably, succinate dehydrogenase activity is not detectable in bradyzoites and the drug atovaquone, which specifically inhibits cytochrome b in complex III of the respiratory chain, causes bradyzoite differentiation (Denton et al., 1996; Tomavo and Boothroyd, 1995). Together with the observations that NO acts upon the parasite’s unique mitochondrion and that loss of mitochondrial potential is associated with the bradyzoite differentiated state, a model becomes apparent where type II parasites initiate the macrophage-induced production of NO to promote their own differentiation and persistence (Bohne et al., 1994; Toursel et al., 2000).
1.6.4 Bradyzoite differentiation *in vitro*

Studying the interconversion between tachyzoite and bradyzoite is a challenging area of research, particularly *in vivo*. The combination of low parasite abundance and the complexities added by a live host background make the need for an *in vitro* model of *Toxoplasma* infection and differentiation apparent. Over the last few decades, a standardization of *Toxoplasma in vitro* culture and differentiation methods has emerged. For example, parasites are routinely cultured in confluent human foreskin fibroblasts (HFF) because *Toxoplasma* more easily infects monolayers than suspension cells and they can replicate many times in this long and narrow cell type, yielding a high number of parasites with little host cell contamination after host cell lysis (Roos *et al.*, 1994). Also, benefiting from the parasite-specific selectable marker hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPR), it is possible to study stage-specific gene expression in an *in vitro* system (Bohne and Roos, 1997).

Induction of bradyzoite differentiation in tissue culture systems was unreliable and poorly understood in its infancy. While addition of IFNγ or sodium nitroprusside, a chemical NO donor, to tissue culture systems induced bradyzoite formation, they only did so at moderate rates (Bohne *et al.*, 1993; Bohne *et al.*, 1994). However, it had also been shown that certain *Toxoplasma* lineages could spontaneously convert from tachyzoite to bradyzoite *in vitro* even in the absence of immunologic factors, suggesting that the conversion process may be activated by other signals in addition to NO (Dardé *et al.*, 1989). Shortly thereafter, it was revealed that a variety of chemical and physical stimuli could induce bradyzoite development nearly completely, making it possible to reliably study the bradyzoite stage *in vitro* for the first time (Soete *et al.*, 1994). These conditions included
prolonged heat shock at 43°C, increased pH of culture media with concurrent CO₂ reduction to atmospheric levels, or addition of the oxidative stress agent sodium arsenite (Soete et al., 1994). Other studies have identified pyrrole- and pyrrolidine-containing chemicals that induce differentiation through poorly characterized mechanisms (Radke et al., 2006; Conde de Felipe et al., 2008). A systematic analysis has examined the relative proficiency of these alternate methods of inducing bradyzoite conversion (Behnke et al., 2008). Although the trisubstituted pyrrole, termed compound 1, appeared to be the most efficient, increasing the pH of the media with concurrent induction of pyrimidine starvation through the reduction of CO₂ levels has emerged as the most common method for initiating bradyzoite differentiation in vitro (Behnke et al., 2008; Bohne and Roos, 1997).

More recently, studies have focused on determining how Toxoplasma copes with a variety of cellular stresses and how these drive the differentiation of tachyzoites towards bradyzoites. By submitting parasites to a barrage of cellular stresses, it has been suggested that in response to nearly any stress, Toxoplasma initiates a process of translational control (Sullivan et al., 2004; Narasimhan et al., 2008; Joyce et al., 2010; Konrad et al., 2011; Konrad et al., 2013a; Joyce et al., 2013; Konrad et al., 2013b). While under the effects of this generalized translational control mechanism, stress-responsive transcripts are prioritized for translation and this has been suggested to be the driving force behind the tachyzoite to bradyzoite conversion (Joyce et al., 2013).

1.7 Post-transcriptional gene regulation in gene expression

Gene expression is the summation of transcriptional and post-transcriptional events that occur to a particular transcript, as well as the collection of post-translational regulatory
events. Transcriptional gene regulation relies on inducing, suppressing, or otherwise modifying the amount of DNA transcription at a particular genomic locus. While this process is widely used to control gene expression throughout different tissues, developmental stages, and/or under different environmental conditions, transcriptional regulation is a relatively slow process and is insufficient to rapidly and precisely modulate gene expression when compared with the potential finesse that post-transcriptional gene regulation (PTGR) offers (Moore, 2005; Hall et al., 2005; Mair et al., 2006). While PTGR may more commonly be considered to consist of methods for regulating mRNA translation, it is a more general umbrella term encompassing a variety of mechanisms that control the expression of gene products by operating at the transcript level. Importantly, all PTGR mechanisms rely on the interaction between cis-acting elements encoded within the transcript and the trans-acting factors that bind and recognize them (Moore, 2005). Together, the mRNA and the collection of RNA-binding proteins (RBP)s form the messenger ribonucleic particle (mRNP), a dynamic complex that changes as the transcript is shuttled from one location to the next in the cell (Moore, 2005). The components of the mRNP at any given time dictate which biological processes the transcript will undergo at any given time (Moore, 2005). Therefore, it is important to note that decisions made early in the mRNA’s biogenesis directly affect its participation in downstream regulatory events by influencing the content of its cis-acting elements and its subsequent ability to recruit certain RBPs (Moore, 2005; Darnell, 2013). Below follows a description of mRNA maturation and cap-dependent translation to serve as a reference point from which to build on downstream regulatory events.
1.7.1 mRNA maturation and nuclear PTGR mechanisms

Transcript maturation and the mechanisms of nuclear PTGR are intimately linked, since each step in the maturation process leaves a trace on the transcript either in the form of altered nucleotide composition and / or through deposition of RBPs at specific locations. The first step in maturation is the co-transcriptional introduction of one of the most universally recognizable cis-acting elements, the 5’ 7-methylguanosine cap (Topisirovic et al., 2011). The 5’ cap is added by a 5’ to 5’ linkage to the 20-30 nucleotide long nascent transcript through the sequential actions of the enzymes nucleotide triphosphatase, guanylyltransferase, and N7-methyltransferase (Topisirovic et al., 2011). The 5’ cap serves as protection from the many 5’ to 3’ exonucleases in the cell and as a key recognition point for a variety of cap-binding proteins including the nuclear cap-binding complex (nCBC; Topisirovic et al., 2011; Moore, 2005; Carmody and Wente, 2009).

The next major maturation event, occurring within thirty seconds of transcription, is the addition of the 3’ poly(A) tail to the pre-mRNA (Darnell, 2013). To gain a poly(A) tail, the cleavage and polyadenylation specificity factor first binds the encoded polyadenylation signal and, after inducing an endonucleolytic cut, recruits nuclear poly(A) polymerase (Gruber et al., 2014). In metazoans, this template-independent polymerase generates a tail of between 200 to 250 adenosines which is then coated by nuclear poly(A)-binding protein (PABP; Gruber et al., 2014). In transcripts containing multiple polyadenylation sites, the usage of alternative polyadenylation sites plays an important role in modulating transcript translatability and stability by mediating the inclusion or exclusion of cis-acting elements, including potential microRNA-binding sites, within its 3’ untranslated region (UTR; Gruber et al., 2014; Darnell, 2013). This PTGR mechanism likely occurs in many T. gondii
transcripts. For example, the \textit{DHFR-TS} 3’UTR varies in length from approximately 250 to 900 nucleotides depending on which of fourteen polyadenylation sites is used (Matrajt \textit{et al.}, 2004).

The final step to generate a mature transcript is the splicing out of introns. While some splicing can occur while the nascent pre-mRNA is still being transcribed, the majority occurs after RNA polymerase II has finished transcribing and polyadenylation is complete (Darnell, 2013). Splicing of an intron involves the participation of the 5’ donor and 3’ acceptor sites, and an adenosine branch point residue located upstream of a polypyrimidine tract within the intron (Suvorova and White, 2014). The components of the spliceosome are recruited through interaction with nCBC and sequentially mediate cleavage at the 5’ donor site, lariat formation through the transesterification of the 5’ donor site guanosine to the branch point adenosine, and the cleavage of the 3’ acceptor site with concurrent linkage of 5’ and 3’ exons (Topisirovic \textit{et al.}, 2011; Darnell, 2013; Suvorova and White, 2014). A successful splicing event yields a correct linkage between the 5’ and 3’ splice sites and the deposition of the exon junction complex (EJC) approximately 20 to 30 nucleotides upstream of each exon-exon junction (Moore, 2005; Carmody and Wente, 2009). The alternative use of splice sites is a mechanism that can be used to generate multiple protein isoforms through the use of a single genomic locus by including or excluding selected exons and / or introns into a mature transcript (Hassan \textit{et al.}, 2012). However, alternative splicing can also be used to selectively post-transcriptionally downregulate gene expression. For example, if an EJC is deposited onto the 3’UTR of a transcript, it serves as a marker of an improperly spliced transcript and will signal its demise through nonsense mediated decay in the cytoplasm (Moore, 2005). Interestingly, while
*Toxoplasma* contains functional splicing machinery and over 75% of predicted genes contain introns, few studies have examined alternative splicing as a means of PTGR (Suvorova et al., 2013; Suvorova and White, 2014). Despite this, sporadic reports have shown that some transcripts, including the widely used selectable marker *HXGPRT* are alternatively spliced (Hasan et al., 2012; White et al., 2000).

The final step of nuclear PTGR is the export of the mature transcript to the cytoplasm through the nuclear pore complex. In most cases, this requires the recruitment of the transcription-export complex which, in metazoans, is indirectly recruited through the 5’ cap and EJC deposited on spliced transcripts (Carmody and Wente, 2009). Alternatively, if a transcript is improperly processed, it is degraded by the nuclear exosome (Carmody and Wente, 2009). Shuttling of the mature mRNP occurs through the nuclear pore complex likely through a 5’ to 3’ threading process and the emergent transcript can be directly recruited to the translational machinery, even before it has entirely entered the cytoplasm (Moore, 2005; Carmody and Wente, 2009).

1.7.2 Cap-dependent translation

The export of the mRNP from the nucleus into the cytoplasm signals a dramatic remodeling of protein complement associated with the transcript. For example, after an initial round of translation, the nCBC and the nuclear PABP are exchanged for their cytoplasmic equivalents, priming the transcript for translation (Moore, 2005). Cap-dependent translation is the most common mode by which eukaryotic organisms initiate protein production (Figure 1.3; reviewed in Sonenberg and Hinnebusch, 2009). Under optimal conditions, cap-dependent translation begins with the recognition of the 5’ cap by
a collection of eukaryotic initiation factors (eIFs) termed the eIF4F complex, which is composed of the cap binding protein eIF4E, the DEAD-box helicase eIF4A, the eIF3-interacting eIF4B, and the eIF4G scaffold (Sonenberg and Hinnebusch, 2009). Notably, eIF4E is one of the most highly regulated proteins involved in translation and many organisms produce multiple isoforms that can have specialized roles in certain tissues, in developmental phases, or in stress response (Topisirovic et al., 2011; Martínez-Silva et al., 2012). Furthermore, in certain cases, different eIF4E and eIF4G isoforms can combine to form specialized eIF4F complexes that show transcript specificity by either favouring short and unstructured 5’UTRs or promoting the translation of transcripts containing longer, structured 5’UTRs (Mayberry et al., 2011; Martínez-Silva et al., 2012). Intriguingly, Toxoplasma encodes at least two eIF4E isoforms and multiple proteins that contain canonical eIF4G domains, however these remain uninvestigated (Xia et al., 2008).

Upon binding of the 5’ cap by eIF4F, the mRNA circularizes through the interaction between eIF4G and PABP in the closed loop model of eukaryotic translation (Figure 1.3; Sonenberg and Hinnebusch, 2009). This facilitates the recruitment of the pre-initiation complex which is composed of the small 40S ribosomal subunit, eIFs 1, 1A, 3, 5, and the ternary complex (Sonenberg and Hinnebusch, 2009). The ternary complex contains the initiator transfer RNA, Met-tRNAi, and eIF2-GTP, which is responsible for recruiting Met-tRNAi to the pre-initiation complex (Sonenberg and Hinnebusch, 2009). After the pre-initiation complex is recruited to the mRNA, it commences processively scanning the 5’UTR until perfect complementarity is achieved between the AUG start codon and the anti-codon contained within the Met-tRNAi (Hinnebusch, 2011). In particularly long or highly structured 5’UTRs, scanning is facilitated through the ATP-dependent helicase
activity eIF4A and other helicases (Hinnebusch, 2011). Most start codons are flanked by an adenosine three nucleotides upstream and a guanosine four nucleotides downstream of the translational start site and if these conditions are not met a downstream AUG can be selected as the preferred start codon in a process known as leaky scanning (Kozak, 1987; Hinnebusch, 2011). This sequence preference is conserved in *T. gondii* and thought to be important for mediating contacts between these residues and other eIFs (Seeber, 1997; Hinnebusch, 2011). Recognition of the start codon triggers the irreversible eIF2-mediated hydrolysis of GTP, the release of eIFs, and recruitment of the large 60S ribosomal subunit (Sonenberg and Hinnebusch, 2009). Translation of the mRNA then proceeds through successive rounds of translational elongation until reaching the stop codon, a termination signal that signals for the dissociation of the ribosome.
An assembled eIF4F complex binds the mRNA 5’cap causing its circularization. The pre-initiation complex is then recruited and scanning of the 5’UTR is conducted until start codon recognition. eIF2 mediated GTP hydrolysis occurs, inducing the recruitment of the 60S large ribosomal subunit and departure of most eIFs. Regulation of translational initiation occurs by phosphorylation events mediated by the TOR and eIF2α kinases. Adapted from Sonenberg and Hinnebusch, 2007.
1.8 Translational control and translational repression

The selective control of translation is an important regulatory mechanism for controlling gene expression. General modes of translational control operate under stressful conditions and permit the widespread translational repression of most transcripts while promoting the translation of a limited number of stress-responsive transcripts. In contrast, transcript-specific translational control selectively withdraws transcripts from the translational pool and can be commonly seen on a case by case basis operating under optimal growth conditions, stressful conditions, or throughout developmental phases. However it is important to note that none of the general or transcript-specific translational repression mechanisms are mutually exclusive.

1.8.1 Translational control mediated by target of rapamycin

In principle, the many steps of eukaryotic translation are subject to regulation, however, the most common regulatory events control the rate-limiting steps of translational initiation (Gastens and Fischer, 2002; Sonenberg and Hinnebusch, 2009). A key regulatory mechanism in higher eukaryotes rests upon modulating the ability of eIF4F complex to bind the 5’ cap, which is accomplished by the kinase target of rapamycin (TOR; Figure 1.3; Sonenberg and Hinnebusch, 2009). In metazoans, under optimal conditions that include the presence of mitogens and growth factors, TOR is activated by the kinase AKT which itself is activated through PI3K (Hay and Sonenberg, 2004). TOR is responsible for either directly or indirectly phosphorylating many components of the eIF4F complex (Hay and Sonenberg, 2004). Through the downstream effector ribosomal protein S6 kinase, TOR contributes to the phosphorylation of eIF4B which serves to increase the recruitment the small ribosomal unit to the pre-initiation complex and to promote the helicase activity of
eIF4A, allowing for increased 5’UTR scanning (Hay and Sonenberg, 2009; Baird and Wek, 2012). Furthermore, while the phosphorylation of eIF4G has unknown consequences, that of eIF4E-binding proteins (4E-BPs) decrease their affinity for the cap-binding eIF4E, thereby allowing the latter to participate in the eIF4F complex (Hay and Sonenberg, 2004; Sonenberg and Hinnebusch, 2009). In contrast, upon amino acid starvation TOR is inactivated, reducing cap-dependent translation and causing a generalized translational repression (Hay and Sonenberg, 2004; Sonenberg and Hinnebusch, 2009; Baird and Wek, 2012).

Interestingly, certain mRNA subsets appear to be preferentially repressed while others are more favourably translated under TOR inactivation (Hay and Sonenberg, 2004; Baird and Wek, 2012). The loss of functional eIF4B decreases eIF4A helicase processivity, reducing the ability of the pre-initiation complex to scan through and melt strong secondary structures in transcripts containing long and structured 5’UTRs (Baird and Wek, 2012). TOR inactivation also results in the sequestration of eIF4E by hypophosphorylated 4E-BPs and causes a global inhibition of 5’ cap recognition and translational repression (Figure 1.3; Sonenberg and Hinnebusch, 2009; Baird and Wek, 2012). However, transcripts whose 5’ most nucleotides consist of an oligopyrimidine tract are particularly prone to TOR-mediated translational repression. Notably, interactions between eIF4E and the transcript are formed through both the 7-methylguanosine cap and the first nucleotide of the transcript and it has been suggested that eIF4E has a comparatively reduced affinity for mRNAs with 5’ terminal oligopyrimidine tracts (Topisirovic et al., 2010; Thoreen et al., 2012). Consequently, even a mild reduction in 4E-BP phosphorylation levels can trigger a dramatic translational reduction for these transcripts (Thoreen et al., 2012).
In contrast, stress-induced translational activation is seen in a subset of mRNAs that contain an internal ribosomal entry site (IRES) within their 5’UTR (Sonenberg and Hinnebusch, 2009; Martínez-Salas et al., 2013). IRESes are cis-acting elements that are diverse in both structure and sequence composition and are found in a variety of metazoan transcripts whose gene products are important for mediating the stress response, such as transcription factors (Martínez-Salas et al., 2013; Sonenberg and Hinnebusch, 2009). When under cellular stress, context-specific IRES trans-acting factors are recruited to their cognate IRESes and serve to stabilize and / or remodel them to promote the direct recruitment of the translational machinery (Martínez-Salas et al., 2013). This mechanism allows these transcripts to bypass the generalized cap-dependent translational repression that occurs under stresses such as amino acid starvation. Interestingly, TOR has also been shown to be responsive to amino acid starvation in Toxoplasma (Ghosh et al., 2011). However, while it has been shown to be a key regulator in the stress-induced autophagic loss of T. gondii’s unique mitochondrion, its influence on the two eIF4Es and role in translational control remain unexplored (Ghosh et al., 2011; Xia et al., 2008).

1.8.2 Translational control mediated by eIF2α phosphorylation

Another stress-induced generalized translational control mechanism rests upon limiting the amount of ternary complex available for pre-initiation complex assembly (Sonenberg and Hinnebusch, 2009). After eIF2 hydrolyses GTP and dissociates from the pre-initiation complex, it must have its GDP exchanged for a GTP through the action of the nucleotide exchange factor eIF2B before being able to participate in another round of translational initiation (Figure 1.3; Sonenberg and Hinnebusch, 2009). Various stressful stimuli induce the phosphorylation of the eIF2α subunit and this increases its affinity for
eIF2B, thereby turning it into a competitive inhibitor of the nucleotide exchange factor (Sonnenberg and Hinnebusch, 2009). In turn, this reduces the turnover of eIF2-GDP, limiting the pool of ternary complex available for cap-dependent translational initiation and causes a generalized translational repression (Sonnenberg and Hinnebusch, 2009). Phosphorylation of eIF2α occurs at position Ser51 in yeast and humans and can be performed by one of four mammalian eIF2α kinases, depending on the particular cellular stress (Sonnenberg and Hinnebusch, 2009). The eIF2α kinases HRI, PKR, PERK, and GCN2 are activated by low haem concentration or oxidative stress, the presence of long double-stranded viral RNA, accumulation of unfolded proteins in the ER lumen, and amino acid starvation, respectively (Sonnenberg and Hinnebusch, 2009; Yamasaki and Anderson, 2008; Anderson and Kedersha, 2008). This mechanism is also conserved in T. gondii, with stressful stimuli inducing phosphorylation of the cognate Ser71 through the activity of stress-responsive eIF2α kinases (Sullivan et al., 2004). T. gondii encodes four eIF2α kinases termed TgIF2K-A through -D (Narasimhan et al., 2008). TgIF2K-A is a homolog of PERK and is activated during conditions causing ER stress (Narasimhan et al., 2008). Like HRI, TgIF2K-B is activated upon oxidative stress and likely other forms of cytoplasmic stress (Narasimhan et al., 2008). Two GCN2-like paralogues are expressed; TgIF2K-C mediates the intracellular stress response under amino acid starvation while TgIF2K-D acts solely during the extracellular phase of the lytic cycle (Konrad et al., 2013b; Konrad et al., 2011; Joyce et al., 2010).

While eIF2α phosphorylation is a powerful general repressor of translation, a subset of transcripts that contain one or a series of small open reading frames upstream of the main start codon (uORF)s actually see their translation increased under these conditions
Most commonly, uORF-containing transcripts encode stress-responsive proteins such as transcription factors and have their translation suppressed under optimal growth conditions (Sonenberg and Hinnebusch, 2009; Baird and Wek, 2012). Under normal growth conditions, uORFs mediate the suppression of the main protein by one of two mechanisms depending on their number and positioning within the 5’UTR (Baird and Wek, 2012). In the first mechanism, an out of frame uORF overlaps the main start codon (Baird and Wek, 2012). The ribosome translates the small peptide and dissociates, having bypassed the main start codon (Baird and Wek, 2012). In the second mechanism, a series of uORFs positioned within the 5’UTR is used to suppress translation of the main ORF by successively promoting dissociation of the ribosome as it passes each stop codon (Baird and Wek, 2012). In contrast, under conditions of stress, uORF-containing transcripts benefit from the reduced amount of ternary complex available to promote their translation (Baird and Wek, 2012). After translating the first uORF, the small ribosomal subunit can either dissociate or resume scanning the 5’UTR in the absence of ternary complex (Baird and Wek, 2012). Since ternary complex is in low abundance, the scanning small ribosomal is more likely to scan through proximal inhibitory start codons and re-initiate translation at downstream start codons, thereby promoting translation of the main protein in these systems (Baird and Wek, 2012).

As in higher eukaryotes, generalized translational repression mediated through eIF2α phosphorylation induces the preferential translation of a subset of stress responsive transcripts in Toxoplasma and, if kept in a phosphorylated state either through inhibiting its dephosphorylation or by maintaining various stressful conditions, bradyzoite
development ensues (Yamasaki and Anderson, 2008; Joyce et al., 2013; Konrad et al., 2013a; Sullivan et al., 2004; Narasimhan et al., 2008). Included among the subset of preferentially translated mRNAs are bradyzoite surface antigens, heat shock proteins, and a subset of uORF-containing AP2 transcription factors (Joyce et al., 2013). This underlines that the stress-dependent modulation of cap-dependent translation is key to the bradyzoite transition process by offering an interesting link between stress-induced translational control and the extensive stage-specific transcriptional regulation known to be important for bradyzoite development. Further research in this field will likely yield intriguing results.

1.8.3 Transcript-specific translational repression

General translational control mechanisms can account for large changes to gene expression in response to cellular stresses. However, many transcripts are post-transcriptionally regulated under optimal or stressful conditions or throughout developmental phases through the use of specific regulatory RBPs. Although the partners involved vary on a case by case basis, all transcript-specific regulatory mechanisms rely on the interaction between cis-acting elements generally encoded within the 5′- or 3′UTR of the transcript and trans-acting factors which either bind the RNA directly or through an intermediate. At any given time, the collection and placement of trans-acting factors along the mRNP dictate the localization, stability, and translational competency of the transcript (Moore, 2005).

Targeting an estimated 60% of the transcriptome in humans, the microRNA (miRNA) system is one the most widely used mechanisms to target transcripts for
withdrawal from the translational pool within the cell (Friedman et al., 2009; Carthew and Sontheimer, 2009). miRNAs are derived from RNA polymerase II-derived transcripts or from spliced out intronic sequences (Carthew and Sontheimer, 2009). miRNA precursors, which form RNA hairpins, are processed first in the nucleus by the double-stranded ribonuclease Drosha and, after nuclear export, by the related cytoplasmic nuclease Dicer, generating a 22 nucleotide RNA duplex with 3’ dinucleotide overhangs (Carthew and Sontheimer, 2009). This RNA duplex is then loaded onto the ribonuclease Argonaute (AGO), which preferentially incorporates whichever one of the two strands has weaker 5’ end base pairing (Carthew and Sontheimer, 2009). The loaded miRNA is used by AGO as a guide to target transcripts destined for PTGR (Carthew and Sontheimer, 2009). Targeting is mediated through the use of the miRNA’s seed sequence, consisting of nucleotides 2-8, which binds through Watson-Crick base pairing to a generally 3’UTR located cis-acting element on target transcript (Carthew and Sontheimer, 2009). Interestingly, approximately 40% of human transcripts have differential polyadenylation site selection which can mediate the inclusion or exclusion of miRNA binding sites within the 3’UTR, offering a direct link between nuclear PTGR and downstream gene regulatory events (Darnell, 2013).

Generally, the binding of AGO to a transcript has an inhibitory effect on its translation through one of two mechanisms. If the miRNA-target interaction consists of perfect base pairing and if the AGO isoform is catalytically active, the target transcript is cleaved, inducing its degradation through the actions of cellular exonucleases (Carthew and Sontheimer, 2009). However, if the miRNA-target interaction contains base pair mismatches outside of the seed sequence or if the AGO isoform is catalytically inactive,
cleavage does not occur and the transcript is generally withdrawn from the translational pool (Carthew and Sontheimer, 2009).

While the importance of miRNA-directed PTGR is well documented in plants and metazoans, its importance in protozoan parasites as an endogenous regulatory mechanism is only just beginning to emerge. It has recently been shown that *Toxoplasma* produces many small regulatory RNAs with the characteristics of miRNAs, some of which are strain-specifically regulated, suggesting that a large number of genes are likely targeted to be post-transcriptionally regulated (Braun *et al.*, 2010). Also, a functional RNA silencing machinery is expressed, however it is somewhat modified compared to most widely characterized systems (Braun *et al.*, 2010; Musiyenko *et al.*, 2012). While perfect miRNA-target matches can be cleaved by AGO, imperfect matches are also cleaved through the actions of a Tudor nuclease, which is recruited to AGO through a methylation-dependent mechanism (Musiyenko *et al.*, 2012).

Many more cases of transcript-specific translational repression occur through the direct interactions between *cis*-acting elements and RBPs. These act to withdraw the transcript from the translational pool while serving as key factors in the remodeling of the mRNP complex. One of the most commonly studied families of *cis*-acting elements that mediate translational control in mammalian cells are AU-rich elements (ARE)s which consist of overlapping AUUUA motifs (Brooks and Blackshear, 2013). Typically located within 3’UTRs, AREs have been shown to bound by a variety of translational repressors such as tristeraprolin (TTP) which generally induces the degradation of the transcript (Brooks and Blackshear, 2013; Arribas-Layton *et al.*, 2013). AREs are also capable of
binding other factors such as the T cell internal antigen-1 (TIA1), which is an RBP involved in the translational repression and storage of transcripts under stress (Cruz-Gallardo et al., 2013). Furthermore, TIA1 also binds 5’oligo pyrimidine tracts, which represents a major category of repressed transcripts upon stress-induced TOR-inactivation (Cruz-Gallardo et al., 2013; Thoreen et al., 2012).

A large amount of research on transcript-specific translational repression has been conducted on oocytes and the early embryogenesis stages of development. During these stages, transcription is non-functional and all protein translation derives from stored maternal transcripts, therefore offering an opportunity to study translational control in a biological system without the background signal potentially generated from ongoing transcription (Vardy and Orr-Weaver, 2007; Barckmann and Simonelig, 2013). In Drosophila, a key upstream regulator in the developmental gene expression cascade is the RBP SMAUG, which binds a structural cis-acting element in the 3’UTR of its target transcript NANOS (Vardy and Orr-Weaver, 2007; Crucs et al., 2000). This element consists of bipartite RNA hairpins with terminal CNGGN loop motifs extending from a central stem and underlines that RNA structure plays an important role in its function (Crucs et al., 2000; Barckmann and Simonelig, 2013). In order to confer translational repression, SMAUG recruits the 4E-BP CUP which competes with eIF4G in order to inhibit translational initiation and deadenylation (Nelson et al., 2004; Vardy and Orr-Weaver, 2007). Interestingly, the NANOS protein itself is also involved in the translational repression of transcripts containing the nanos response element (NRE), a UC-rich element consisting of two central UUGU motifs (Vardy and Orr-Weaver, 2007; Zamore et al., 1997). HUNCHBACK contains a NRE within its 3’UTR and is bound by PUMILIO, a PUF family
protein which is responsible for recruiting the other members of the NRE complex, NANOS and BRAIN TUMOR (Vardy and Orr-Weaver, 2007). In turn, the NRE complex recruits the deadenylation complex and d4EHP, an inhibitory cap-binding protein with high homology to eIF4E that lacks the capacity to interact with eIF4G (Vardy and Orr-Weaver, 2007).

PUF family members have been shown to play roles as translational repressors involved in the controlling of developmental stage progression in a variety of organisms throughout the evolutionary tree including apicomplexan parasites (Gomes-Santos et al., 2011). In *Plasmodium* female gametocytes, the PUF2 protein binds a large number of translationally repressed transcripts, including those encoding the major ookinete surface antigens P25 and P28, through a PUF-binding element which consists of a UGUX\textsubscript{3-5}UA motif located in either the 5’ or 3’UTR of the target transcript (Miao et al., 2013; Braks et al., 2008). Translational repression of these transcripts is relieved upon conversion to zygotes, where transcription is restricted after gamete fertilization (Mair et al., 2006). Interestingly, PUF2-mediated translational repression also acts in other stages of the parasite’s life cycle. Disruption of PUF2 in the salivary gland sporozoite stage of development causes the premature translation of repressed transcripts, including transcription factors (Miao et al., 2013). In turn, large scale alterations in the parasite’s transcriptome occur resulting in the premature conversion to the mammalian liver stage of the parasite while still in the mosquito (Miao et al., 2013). These changes point to *Plasmodium* PUF2 serving as a master regulator of developmental progression by working as a stage-specific translational repressor (Miao et al., 2013). Intriguingly, the *Toxoplasma* PUF2 homolog, PUF1, is capable of binding *Plasmodium* PUF-binding elements in vitro,
is upregulated in bradyzoites, and preferentially translated under stressful conditions, raising the interesting possibility of it serving a similar function in mediating *Toxoplasma* development (Liu *et al*., 2014; Joyce *et al*., 2013).

1.9 Fate of translationally repressed mRNAs

The translational machinery and repressor proteins remain in competition throughout the life of the transcript since, under the right conditions, repressed transcripts can re-enter the translational pool (Anderson and Kedersha, 2008). However, by limiting access to the translational machinery, a translational repressor can remodel the mRNP components by revealing novel sites on the transcript for RBP interactions and by directly promoting the association of other RBPs involved in mediating a transcript’s turnover, long-term maintenance, and/or sub-cellular localization (Vardy and Orr-Weaver, 2007; Arribas-Layton *et al*., 2013; Hooper and Hilliker, 2013). Although the processes of translational repression can be separated from its downstream fate, withdrawal from translation typically induces the physical movement of the transcript into sub-cellular foci where they are processed either for degradation, short-term storage, or long-term storage and maintenance (Anderson and Kedersha, 2008).

1.9.1 Processing bodies and mRNA degradation

In many cases, RBPs that induce translational repression also promote the association of machinery involved in the transcript’s degradation. For example, the translational repressors TTP and PUF proteins have been shown to interact with the machinery involved in the removal of the poly(A) tail and 5’cap (Godwin *et al*., 2013; Arribas-Layton *et al*., 2013). Typically mRNA decay occurs in a series of steps, the first of which is the
degradation of its poly(A) tail through the actions of the 3’ to 5’ poly(A) specific ribonuclease or a functional equivalent (Godwin et al., 2013). Notably, this step is reversible and while the shortening of the poly(A) inhibits the association of PABP with eIF4G, its lengthening by a cytoplasmic poly(A) polymerase is key to re-initiation of translation (Sonenberg and Hinnebusch, 2009; Godwin et al., 2013). Maintaining a short poly(A) tail is a key method by which transcripts are kept in a state of translational quiescence and the balance of tail shortening versus tail lengthening helps dictate the translational competence throughout development (Godwin et al., 2013). Deadenylation greatly enhances the second step in mRNA decay, the removal of the 5’ cap from the transcript (Arribas-Layton et al., 2013). The decapping complex, consisting of DCP1 and DCP2, catalyses the removal of a 7-methyl-GDP from the transcript, leaving a monophosphate 5’ terminal nucleotide (Arribas-Layton et al., 2013). The decapping machinery can be specifically recruited to transcripts through the use of RBP adaptors such as TTP, PUF proteins, and the yeast DEAD box helicase DHH1 (Arribas-Layton et al., 2013).

Aside from serving as recognizable components to promote translation, the 5’cap and poly(A) tail also play an important role in protecting the transcript from the ribonucleases present in the cell (Topisirovic et al., 2011; Nagarajan et al., 2013; Chlebowskii et al., 2013). Removal of these elements subjects the transcript to 3’ to 5’ degradation as it is threaded into the exosome as well as to the 5’ to 3’ exonuclease activity of XRN1. These exonucleases also act in other pathways with the exosome being responsible for degrading transcripts through nonsense mediated decay and removing the 5’ fragment of AGO-
cleaved transcripts while the XRN1 exonuclease degrades the 3’ fragment (Chlebowski et al., 2013; Nagarajan et al., 2013).

In most eukaryotes, the mRNA degradation machinery is preferentially localized in cytoplasmic foci called processing bodies (P-bodies; Anderson and Kedersha, 2008). In metazoans, P-bodies contain a key scaffolding protein, GW182, while the decapping and deadenylase machinery are characteristic components of P-bodies in all organisms (Moser and Fritzler, 2010; Eulalio et al., 2007; Anderson and Kedersha, 2008). They also contain translational repressor proteins such as PUF proteins, TTP, DHH1, and AGO while excluding PABP and translational initiation factors (Anderson and Kedersha, 2008; Godwin et al., 2013; Hooper and Hilliker, 2013). While P-bodies can be present under normal growth conditions, they have been shown to increase in aggregation under stressful conditions (Anderson and Kedersha, 2008). Interestingly, it has been suggested that the stress-induced increase in P-body formation is a result rather than a mechanism of increased transcript decay, as degradation proceeds unimpeded if P-body assembly is inhibited (Anderson and Kedersha, 2008; Eulalio et al., 2007).

1.9.2 Stress granules and short term storage of transcripts

Stress granules (SGs) are cytoplasmic foci that form rapidly in response to eIF2α phosphorylation and consist of transcripts stalled in the process of translational initiation (Anderson and Kedersha, 2008). While SGs and P-bodies share many components including translational repressors like TIA1, TTP, AGO, DHH1, and SMAUG as well as mRNA, SGs are uniquely characterized by the inclusion of PABP, various eIFs and the small 40S ribosomal subunit consistent with its role in aggregating stalled translational
machinery (Anderson and Kedersha, 2008). Furthermore, in contrast to P-bodies and consistent with SGs containing primarily stalled initiation transcripts, mRNAs within SGs are polyadenylated (Stoecklin and Kedersha, 2013).

In contrast to most other large cytoplasmic mRNP granules, the aggregation of SGs is fairly well defined (Moser and Fritzler, 2010; Anderson and Kedersha, 2008). The decrease in available ternary complex in response to eIF2α phosphorylation drastically reduces the rate of translational initiation, while translational elongation and termination proceeds unimpeded (Anderson and Kedersha, 2008). This causes the run off of translating ribosomes and the accumulation of stalled initiation complexes (Anderson and Kedersha, 2008). The removal of the translational machinery from the transcript at this step may allow for increased RBP interactions causing transcript-specific translational repression (Arribas-Layton et al., 2013). Subsequently, mRNP aggregation and nucleation occurs, likely through the actions of prion-like domains in certain RBPs, such as those seen in TIA1 and some RNA helicases (Anderson and Kedersha, 2008; Voronina et al., 2011). This aggregation then intensifies as more RBP-mRNA and protein-protein interactions occur (Anderson and Kedersha, 2008). Notably, the interactions mediating SG assembly are thought to be fairly weak and SGs have been said to have a liquid-like structural fluidity, with RBP components such as TIA1, TTP, and PABP rapidly shuttling in and out of SGs (Voronina et al., 2011, Stoecklin and Kedersha, 2013; Moser and Fritzler, 2010; Anderson and Kedersha, 2008). Furthermore, inhibition of translational elongation depletes SGs formation, suggesting that the dynamic SG components are in equilibrium with the translational machinery (Anderson and Kedersha, 2008).
While the identity of many SG-associated RBPs is known, the identity of transcripts that accumulate in SGs upon stress is less well established. However, studies have shown that inclusion of transcripts within SGs is selective and mediated by \textit{cis}-acting elements (Anderson and Kedersha, 2008; Hooper and Hilliker, 2013). For example, housekeeping transcripts are preferentially recruited to SGs while transcripts corresponding to stress-responsive genes get excluded (Anderson and Kedersha, 2008). Notably, while the latter category consists of transcripts that are translationally permissive under stress, the former members are translationally repressed. This has led to the theory that SGs are essential to the relief of cellular stress by allowing stress-responsive transcripts to receive preferential access to the limited amount of functional translational machinery (Kedersha and Anderson, 2002; Yamasaki and Anderson, 2008).

The existence of stress-induced mRNA cytoplasmic foci with the appearance and characteristics of SGs has recently been found in \textit{T. gondii} (Lirussi and Matrajt, 2011). These granules appear exclusively during the extracellular phase of the parasite, are phospho-eIF2a dependent, and contain both PABP and the \textit{Toxoplasma} homolog of yeast DHH1, but do not include AGO (Lirussi and Matrajt, 2011; Cherry and Ananvoranich, 2014). Notably, immunocytochemistry experiments have shown that both PABP and DHH1 completely overlap the signal generated by a fluorescent oligo-dT probe (Lirussi and Matrajt, 2011; Cherry and Ananvoranich, 2014). Another study has found granules in extracellular parasites which are marked by the presence of the RBPs ALBA1 and ALBA2, with both proteins also showing perfect overlap with polyadenylated mRNA (Gissot \textit{et al.}, 2013). However, these granules have a distinct perinuclear localization and co-immunoprecipitation studies using a mix of intracellular and extracellular tachyzoites have
shown that ALBA1 is associated with AGO as well as a host of translational machinery (Gissot et al., 2013). Furthermore, ALBA1 has been implicated in the translational activation of transcripts (Gissot et al., 2013). While different experimental conditions and / or the dynamics of the partners involved may account for these discrepancies, further research into the composition and dynamics of stress-induced polyadenylated mRNA-containing granules is needed.

Interestingly, the formation of mRNA-containing granules has been shown to be important for *Toxoplasma* to successfully transition from the extracellular to the intracellular environment (Lirussi and Matrajt, 2011). Extracellular tachyzoites that were inhibited from forming mRNA-containing granules displayed reduced invasion and growth (Lirussi and Matrajt, 2011). It was suggested that these granules served to promote the sequestration of “intracellular” transcripts during the extracellular period, therefore giving preference to “extracellular”-responsive transcripts and preparing for intracellular growth, in turn increasing the fitness of the parasites (Lirussi and Matrajt, 2011). This suggests a short-term salvage strategy is used by the parasite to selectively withdraw transcripts from the translation pool under stressful conditions and maintain them for use in a later developmental phase and is consistent with the hypothesized role of canonical stress granules (Lirussi and Matrajt, 2011; Kedersha and Anderson, 2002; Yamasaki and Anderson, 2008; Moore, 2005).

1.9.3 Germ granules and long term storage of transcripts

It has been suggested that in *Toxoplasma*, stress-induced generalized translational repression provokes the degradation of translationally repressed transcripts through mRNA
decay mechanisms (Joyce et al., 2013). While this is likely the case in the vast majority of repressed transcripts, it raises the question as to what happens to those transcripts that lack translational competency and yet are maintained at appreciable levels in bradyzoites, which can lie dormant for decades before initiating stage conversion to tachyzoites. This observation is suggestive of a developmentally-controlled mechanism that selectively reduces the mRNA turnover of certain transcripts while inhibiting their translation.

A common system for studying the long-term maintenance of transcripts and how this relates to developmental cycles in eukaryotes is the oocyte and its transition to the early zygote after fertilization (Vardy and Orr-Weaver, 2007; Barckmann and Simonelig, 2013). Oocytes contain a variety of specialized mRNA-containing granules, some of which are able to stably store transcripts over the period of days to years depending on the organism (Voronina et al., 2011; Hooper and Hilliker, 2013; Schisa, 2012). Importantly, these transcripts appear to be recruited in a transcript-specific manner mediated by the interaction between specific cis-acting elements and trans-acting factors (Voronina et al., 2011; Schisa, 2012). For example, in Drosophila, the RBP BRUNO binds the Bruno response element in the 3′UTR of the OSKAR transcript and recruits CUP, causing its translational repression and recruitment to germ granules, which have been implicated in long-term mRNA storage (Voronina et al., 2011; Barckmann and Simonelig, 2013).

Importantly, while germ granules, P-bodies, and SGs have been shown to share many components, they are distinct entities. In contrast to SGs, germ granules do not contain translational initiation components (Voronina et al., 2011). While germ granules may seem more similar to P-bodies, the former contain a greater amount of transcript-specific
translational repression machinery and does not lead to the decay of transcripts (Voronina et al., 2011; Barkmann and Simonelig, 2013). It has therefore been suggested that germ granules may be a functional hybrid or specialized form of SGs and/or P-bodies (Voronina et al., 2011). Supporting this hypothesis is the fact that shared components can have different functions in these granules. For example, while the homolog of DHH1 in *Drosophila* aids in the recruitment of decay machinery in P-bodies, it is an essential component for the stability of transcripts in germ granules and its loss leads to sterility (Voronina et al., 2011). Also, in mouse oocytes it has been shown that GCN2 kinase is upregulated which causes an increase in eIF2α phosphorylation that is only alleviated after zygote formation (Alves et al., 2009). Notably, this occurs concurrently to the disruption of germ granules and an increase in general translation (Alves et al., 2009). Taken together, these results offer a link between generalized translational repression, germ granule formation, and the long-term storage and maintenance of transcripts as well as between germ granule dissolution, translational activation, and developmental progression in higher eukaryotes.

In *Plasmodium*, similar mRNA-containing granules have been shown to form in female gametocytes (Mair et al., 2006; Mair et al., 2010). It is important to note that in the *Plasmodium* lifecycle, gametocytes reside in mammalian red blood cells and must wait until they are taken up by the mosquito in order to initiate fertilization and progression into zygotes. eIF2α is maintained in a phosphorylated state by the kinase IK4 in gametocytes and transcripts, including the zygote-specific surface antigens *P25* and *P28*, are maintained in a state of translational quiescence (Zhang et al., 2012; Hall et al., 2005). Importantly, these transcripts localize to mRNA granules contingent on a 47 nucleotide U-rich cis-acting
element that can be located in either the 5’- or 3’UTR (Mair et al., 2006; Hall et al., 2005; Braks et al., 2008). More recently, these elements have been shown to be bound by the translational repressor PUF2 in vitro and notably, a PUF2 deletion mutant displayed increased P25 and P28 steady state transcript and protein levels, suggesting that this interaction is essential for their translational repression in vivo (Miao et al., 2013). The maintenance of these mRNA granules depends on the Plasmodium DHH1 orthologue, DOZI (Mair et al., 2010). Similar to metazoan germ granules, disruption of DOZI causes the loss of stored transcripts, including P25 and P28, and the inability to develop into zygotes after fertilization (hence the name Development Of Zygote Inhibited; Voronina et al., 2011; Mair et al., 2010). Although a co-immunoprecipitation study failed to find an association between PUF2 and DOZI in female gametocytes, these data would correlate with a model very similar to that of sexual development in higher eukaryotes and led the authors to suggest that the mRNA granules in Plasmodium female gametocytes act specifically to store translationally repressed transcripts in anticipation of stage conversion, similar to what is seen for maternal transcripts in the germ line of metazoans (Mair et al., 2010).

Interestingly, it appears that this mechanism is not limited to sexual reproduction in apicomplexan parasites. The transmission of Plasmodium from mosquitoes is also marked by a long delay where sporozoites must wait in the mosquito salivary glands until they are delivered to the mammalian host, where they can then enter the next developmental phase in the mammalian liver. Salivary gland sporozoites are under a state of global translational repression mediated by IK2-induced eIF2α phosphorylation and this stage is characterized by polyadenylated mRNA and PABP-containing granules that are needed in order to
maintain their developmental state (Zhang et al., 2010). Furthermore, deletion of PUF2 in salivary gland sporozoites, which normally displays granular localization, causes the release of translationally repressed transcripts, including those encoding transcription factors, and premature differentiation into liver-stage parasites (Gomes-Santos et al., 2011; Müller et al., 2011). The potential involvement of DOZI in sporozoite storage granules remains unexplored, however its involvement may be limited since it is transcriptionally down regulated during this stage (Müller et al., 2011).

These data are consistent with the notion that in apicomplexan parasites, select translationally repressed transcripts are shuttled to storage granules by RBPs based on their recognizable cis-acting elements and maintained until the commencement of the next developmental phase. In contrast to the “just-in-time” model of transcriptional regulation, this “store-and-go” mode of PTGR may be more favourable in certain circumstances since it allows for more rapid translation with less energy input upon developmental transition (Mair et al., 2006).

To date, the existence of bradyzoite-specific mRNA-containing storage granules have not been discovered in Toxoplasma and a comprehensive list of translationally quiescent transcripts has been impossible to generate because, although the identification of most transcriptionally regulated transcripts are known through EST, SAGE, and microarray experiments, a study identifying the bradyzoite proteome, which could be used to generate such a list, has not been performed (Manger et al., 1998; Radke et al., 2005; Cleary et al., 2002). Instead, sporadic reports throughout the literature have demonstrated that while certain genes have their transcripts expressed at appreciable levels in both stages,
their cognate protein is undetectable in bradyzoites. Examples include the RNA-binding DNA repair enzyme \( TgDRE \) and the canonical tachyzoite marker \( LDH1 \) (Dendouga et al., 2002; Yang and Parmley, 1997). Furthermore, a transcriptional pulse-chase assay identified a number of transcripts whose overall mRNA abundance is maintained in bradyzoites despite reduced transcriptional activity suggesting that they are preferentially stored (Cleary et al., 2005). These include transcripts that encode transcriptional and translational machinery as well as the canonical tachyzoite marker \( SAG1 \) (Cleary et al., 2005). Like LDH1, the SAG1 protein is widely used as an immunological marker to identify the tachyzoite stage and is not present in mature bradyzoites (Ferguson, 2004).

These findings, taken together with the role of stage-dependent translational repression suggest that, as seen in \textit{Plasmodium}, a stage-specific mechanism exists in bradyzoites to selectively store and maintain transcripts in anticipation of tachyzoite stage conversion.

1.10 Objectives

Successful tachyzoite and bradyzoite interconversion is crucial for the parasite’s survival and for its ability to be transmitted to a new host. As such, understanding how \textit{Toxoplasma} copes during a stressful insult is an essential part of understanding its fundamental biology. This work aims to further the investigation into the stress response mechanism. In Chapter 2, a demonstrated anti-parasitic agent, securinine, was investigated in an \textit{in vitro} model of \textit{Toxoplasma} infection. Its influence on parasite replication and bradyzoite differentiation is outlined. Using the translationally repressed \( LDH1 \) as a model, Chapter 3 describes the identification and characterization of a small RNA \textit{cis}-acting regulatory element that is responsible for mediating transcript-specific translational repression in bradyzoites. Finally, Chapter 4 outlines efforts made towards identifying the
trans-acting factors associated with the LDH1 transcript while it is undergoing translational repression as well as generally characterizing the mechanism.
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CHAPTER II – *Toxoplasma gondii*: Inhibitory activity and encystation effect of securinine and pyrrolidine derivatives on *Toxoplasma* growth

2.1 Introduction

*Toxoplasma gondii* is a widespread opportunistic pathogen of both veterinary and human importance. In the otherwise healthy individual, the immune system keeps the parasite in the asymptomatic dormant bradyzoite state. However, upon immune system suppression the conversion of bradyzoites to the rapidly growing tachyzoites cause a recurrence of acute infection which may lead to toxoplasmic encephalitis (Montoya and Liesenfeld, 2004). The current treatment for acute toxoplasmosis calls for a combined approach using pyrimethamine and sulfadiazine, both folate inhibitors. In certain circumstances, clindamycin, a protein synthesis inhibitor, may also be indicated. However, the currently available drugs have a variety of side effects including intolerance (Montoya and Liesenfeld, 2004). Also, resistance to some of the classical drugs of treatment has been reported in *T. gondii* (Aspinall et al., 2002), as well as other apicomplexans, most notably pyrimethamine resistant *Plasmodium falciparum* (Mita et al., 2009). It is therefore important to discover novel drugs that act upon a multitude of parasite-specific targets.

Many studies have been launched to investigate currently used drugs for their possible uses as anti-parasitic agents. Jones-Brando *et al.* (2003) showed that a number of drugs acting on the central nervous system (CNS), such as the anti-psychotics haloperidol and olanzapine, exhibit anti-*Toxoplasma* activity. Cortez *et al.* (2008) showed that simvastatin, an anti-hypercholesterolemic statin, inhibits the growth of *T. gondii* in macrophages *in vitro*. Other efforts have turned towards the active ingredients found in traditional Chinese medicines for drug discovery (Jiang *et al.*, 2008; Ke *et al.*, 1990).
One compound that has shown promise is securinine, an alkaloid produced by *Securinega suffruticosa*. As reviewed by Raj and Luczkiewicz (2008), the plant is one of the 50 basic medical plants in traditional Chinese phytotherapy and was used in the treatment of various ailments ranging from rheumatic disease, quadriplegia, paralysis following infectious disease, and impotence. Also, in Russia, where it was initially isolated and used for its GABA antagonistic activity as a CNS stimulant, securinine was referred to as a drug in the 10th Russian Pharmacopoeia-1968, although its drug registry later expired. A previous report has shown that this compound also shows an inhibitory effect against *P. falciparum* (Weenen et al., 1990). More recently, Rana et al. (2010) reported that securinine can preferentially kill cancer cells while protecting normal cells and acts via the p73-dependent apoptotic pathway.

In this study we have analysed the effects of securinine on the proliferation of *T. gondii* in vitro. Moreover, 10 pyrrolidine compounds, which were synthesized from trans-4-hydroxy-L-proline to complete the asymmetric total synthesis of (−)-securinine (Dhudshia et al., 2009), have been selected for the study. Assessing their inhibitory effect, we show that securinine and the pyrrolidine derivatives have unique properties in inhibiting *T. gondii* proliferation in comparison to pyrimethamine, the classical anti-toxoplasmosis drug.

2.2 Materials and methods

2.2.1 Host cell and parasite cultures

All experiments were carried out using human foreskin fibroblasts (HFF) that were cultured using DMEM medium with L-glutamine and high glucose (Invitrogen, Canada),
supplemented with 10% Cosmic calf serum (Hyclone, Logan, Utah), 0.5x antibiotic–antimyocotic (Invitrogen, Canada) in 5% CO₂. Tachyzoites of *T. gondii* strains RHΔHX (NIH AIDS Research and Reference Reagent Program-2857), a commonly used laboratory strain lacking the gene hypoxanthine-xanthine-guanine phosphoribosyl transferase (Donald *et al.*, 1996), and 2F (ATCC-50839) were propagated in HFF grown in MEM medium with L-glutamine (Invitrogen, Canada), supplemented with 1% dialyzed fetal bovine serum (Hyclone) and 0.5x antibiotic–antimycotic.

2.2.2 Inhibition and cytotoxicity assays

Securinine and 10 pyrrolidine derivatives (Figure 2.1) were dissolved in DMSO to obtain a final concentration of 1 mM. Various concentrations of these compounds (0.1–100 µM followed by 1–1000 µM if the compound showed anti-*Toxoplasma* activity but no host cell cytotoxicity in the first range tested) were tested for inhibitory effects on *T. gondii* proliferation using 96-well plates. Fifty freshly released 2F tachyzoites, which express β-galactosidase (McFadden *et al.*, 1997), were inoculated onto 6/8 wells in each column. On day 3, the β-gal substrate, chlorophenol red–β-D-galactopyranoside, was added and the plate was incubated overnight. On the fourth day, cytotoxicity effects of tested compound were measured in the remaining 2/8 wells per column using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) colorimetric assay as previously described (Tominaga *et al.*, 1999). Colour reactions in all wells were read in a Victor3 microplate reader (PerkinElmer). Experiments were performed in triplicate. DMSO (solvent for the stock solution) was also tested for its inhibitory effect and cytotoxicity (data not shown). After normalizing for experimental variations and expressing data in percentage positivity to control for individual experimental variations (Gubbels *et al.*, 2010).
2003), all inhibitory concentrations (IC$_{50}$) and toxic doses (TD$_{50}$) were determined using
the Reed–Muench formula (Reed and Muench, 1938) and the therapeutic index (TI) was
calculated with the formula $TI = \frac{TD_{50}}{IC_{50}}$ (Table 2.1). Calculated partition coefficient
($c\log P$) and partition coefficient ($\log P$) values were obtained using ChemDraw version
11.0 software (Cambridgesoft, Cambridge, MA).
Figure 2.1 Securinine and 10 pyrrolidine precursors generated via the retrosynthesis of (−)-securinine.

The pyrroldinine precursors (Dhudhia et al., 2009) are divided into groups by their anti-Toxoplasma activity. The structurally unrelated pyrimethamine, the classical anti-toxoplasmosis agent, is included for reference.
2.2.3 Tachyzoite growth rate assessment

Both confluent HFF monolayers grown on cover-slips and freshly lysed RHΔHX tachyzoites were harvested and incubated for one hour in the tested compound at their respective IC_{50} (DMSO used as control). After the initial one hour incubation, the parasites were allowed to infect the cover-slips and grow for either 24 or 36 h for the determination of growth inhibition. The cover-slips were subjected to 3% paraformaldehyde fixation and then Hoechst stained (final concentration 10 µM). For each compound, 100 vacuoles were counted from three independent trials. Average vacuole size was determined using the weighted average and doubling time was determined using algorithm provided by http://www.doubling-time.com/compute.php.

2.2.4 Invasion assay

Confluent HFF monolayers were grown on cover-slips and an equal number of extracellular tachyzoites (approximately 7.5 x 10^4) were incubated with parasite culture media in the presence of selected compounds at their respective IC_{50} for 1 h. The parasites were then used to infect the HFFs and allowed to grow for 24 h, after which they were fixed and stained with Hoechst. Fifty randomly chosen microscopic fields of view were then examined and the density of parasitophorous vacuoles per field of view was determined. Each experiment was performed in triplicate.

2.2.5 Extracellular viability assay

Freshly lysed RHΔHX tachyzoites were pelleted and resuspended in parasite culturing media containing the compound of interest at their respective IC_{50} (DMSO for control) for 1, 2, 3, or 24 h. At each time point, tachyzoites were stained with propidium
iodide (final concentration 1 µg/ml; Sigma Aldrich) and placed on a slide. Ten random fields of view were chosen for each tested compound and tachyzoite viability was assessed by propidium iodide staining.

2.2.6 Determination of parasite differentiation

To evaluate the ability of the test compounds to induce parasite differentiation, freshly lysed RHΔHX tachyzoites were used to infect confluent HFF monolayers grown on cover-slips in the presence or absence of tested compounds for 3 days. At least three independent experiments were performed for each compound. Cover-slips were fixed in 3% paraformaldehyde then permeabilized with 0.25% Triton-X in PBS for 10 min and then incubated with 5% BSA in PBS for 1 h. Cover-slips were subsequently incubated with *Dolichos biflorus* lectin conjugated with FITC (Sigma Aldrich) in order to probe for the presence of the cyst wall glycoprotein, a marker of parasite differentiation (Zhang et al., 2001). Hoechst stain was used for localizing the nuclei of parasite and HFF. As control conditions for the detection of differentiation and cyst wall formation, the parasite was grown under alkali conditions (RMPI medium containing 25 mM HEPES, pH 8.3, 5% dialyzed fetal bovine serum) and atmospheric CO$_2$. Slides were mounted and examined with a Leica DMIRB microscope. All images were taken with a cooled Q-Imaging CCD camera using the Northern Eclipse software. One hundred vacuoles from each independent experiment were examined for the presence of cyst wall glycoprotein among vacuoles formed.
2.3 Results and discussion

2.3.1 Cytotoxic and inhibitory effect of securinine and pyrrolidine derivatives

Ideally an anti-microbial compound should have minimal toxicity towards the host while showing high selectivity and toxicity toward microorganisms. Therefore, we first evaluated the cytotoxic effects of securinine and pyrrolidine derivatives on HFFs in comparison to pyrimethamine. Using MTT assays, tested compounds showed varied cytotoxic doses (TD$_{50}$) ranging from 32 to 291 µM (Table 2.1). Securinine was less cytotoxic (TD$_{50}$ = 81 µM) compared to pyrimethamine, the reference compound (TD$_{50}$ = 44 µM). Compounds 1 and 8 were the most cytotoxic with TD$_{50}$ of 32 and 34 µM, respectively, while the other compounds exhibited equal or less cytotoxicity than pyrimethamine. Compound 21 showed the least cytotoxicity with TD$_{50}$ of 291 µM with a high standard deviation.

Using β-gal expressing 2F tachyzoites, we evaluated the anti-Toxoplasma property of securinine and pyrrolidine derivatives. Securinine exhibited an inhibitory effect against T. gondii proliferation with an IC$_{50}$ of 9.8 µM (Table 2.1). Most of the synthetic pyrrolidine precursors of securinine had good activity against T. gondii with IC$_{50}$s in the micromolar range. Based on their inhibitory effect, these pyrrolidine derivatives can be categorized into four groups. Group A contains compounds 1, 3 and 8 with similar IC$_{50}$ values ranging between 3 and 6 µM. Group B includes compounds 4, 5, and 31 showing IC$_{50}$ values ~30 µM. Group C, comprised of compounds 11 and 21, show IC$_{50}$ values of ~60 µM. The fourth group, Group D, made up of compounds 41 and 51, which have negative logP values, did not show any inhibitory effect on parasite proliferation at the tested concentrations (IC$_{50}$ > 100 µM). Since logP values are an indicator of physicochemical properties and reflect
how well a compound will distribute across cell membranes (the higher log$P$ value, the more hydrophobic the compound; Patrick, 2009), their lack of inhibitory effect on $T. gondii$ proliferation could be explained by their inability to cross the host cell membrane. Notably, the most effective compounds in inhibiting $T. gondii$ proliferation, Group A, have the highest calculated log$P$ values (clog$P$ ~5) and the lowest IC$_{50}$ (~5 µM). Group C has log$P$ values less than 1 with IC$_{50}$ > 100 µM and is the least biologically effective. The data thus suggest that the pyrrolidine derivatives showing the most biological activity are those that are most able to diffuse across the cell membrane.

Among the pyrrolidine compounds tested here, we found that the higher the log$P$ value, the lower IC$_{50}$ value. However, this biological and physicochemical relationship cannot be extended to explain the biological effect of securinine (log$P$ = 0.54). This is possibly due to its different structural features. Securinine, with a tricyclic structure, does not retain the pyrrolidine structure, although it is derived from the same trans-4-hydroxy-L-proline precursor (Dhudshia et al., 2009). We thus postulated that securinine inhibits the parasite proliferation using a different mode and / or target than pyrrolidine derivatives.

To evaluate whether tested compounds show promising therapeutic properties, we used the therapeutic index as an indicator to compare securinine and pyrrolidine derivatives to pyrimethamine (TI of 100). All compounds tested here showed much lower therapeutic indices than pyrimethamine (Table 2.1), suggesting that further chemical modifications onto the structure of securinine and pyrrolidine analogs tested here are required to achieve equivalent or better anti-toxoplasmosis activity. Securinine and Group A (compounds 1, 3 and 8) showed the best therapeutic indices (TI = ~9). Compounds 4 and 5 are stereoisomers
and show the same TI of 3. Group C (compounds 11 and 21) showed some inhibitory effect with variable cytotoxicity. Although these compounds are not yet suitable for a commercial development, it is crucial to gain a better understanding of their mode of action. The information will aid future rational drug design of anti-toxoplasmosis agents. We therefore chose to further examine securinine as well as Group A compounds as they displayed the highest anti-\emph{Toxoplasma} activity.

Table 2.1 \emph{T. gondii} inhibitory concentrations (IC$_{50}$) and HFF toxic doses (TD$_{50}$) of securinine and pyrrolidine derivatives in comparison to those of pyrimethamine.

<table>
<thead>
<tr>
<th>Compound</th>
<th>(\log P)</th>
<th>IC$_{50}$(^{a})</th>
<th>TD$_{50}$(^{a})</th>
<th>TI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Securinine</td>
<td>0.54</td>
<td>9.8 ± 1.6</td>
<td>81 ± 20</td>
<td>8</td>
</tr>
<tr>
<td>1</td>
<td>5.4695(^{b})</td>
<td>3.9 ± 1.2</td>
<td>32 ± 14</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>4.8175(^{b})</td>
<td>5.6 ± 1.6</td>
<td>54 ± 17</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>2.92155(^{b})</td>
<td>27.4 ± 17</td>
<td>80 ± 2</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>2.92155(^{b})</td>
<td>26.6 ± 12.2</td>
<td>83 ± 3</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>4.66115(^{b})</td>
<td>3.9 ± 0.6</td>
<td>34 ± 11</td>
<td>9</td>
</tr>
<tr>
<td>11</td>
<td>0.48</td>
<td>63.3 ± 8.4</td>
<td>84 ± 4</td>
<td>1</td>
</tr>
<tr>
<td>21</td>
<td>0.45</td>
<td>60.4 ± 22.7</td>
<td>291 ± 268</td>
<td>-</td>
</tr>
<tr>
<td>31</td>
<td>2.64</td>
<td>27.0 ± 18.5</td>
<td>49 ± 4</td>
<td>2</td>
</tr>
<tr>
<td>41(^{c})</td>
<td>-0.03</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>51(^{c})</td>
<td>-0.21</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>2.69</td>
<td>0.4 ± 0.1</td>
<td>44 ± 2</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^{a}\) Values ± standard deviation.
\(^{b}\) Calculated \(\log P\).
\(^{c}\) compounds not biologically active at tested concentrations (0.1 – 100 μM).

2.3.2 Inhibitory effect of securinine and pyrrolidine derivatives on the parasite lytic cycle

During infection leading to toxoplasmosis, \emph{T. gondii} tachyzoites progress through the lytic cycle, comprised of three distinct phases: invasion of host cells, intracellular replication, and destruction of host cells (Black and Boothroyd, 2000). The destruction of host cells coincides with the egress of tachyzoites which remain extracellular until they
successfully invade new host cells and begin another cycle. We therefore examined various steps of the lytic cycle to further understand the mechanisms by which securinine and Group A compounds affect intracellular growth.

We first examined the effects these compounds had on intracellular replication by treating them with the compounds at their respective IC$_{50}$ or DMSO as control. The parasites were allowed to proceed for either 24 or 36 h post-infection after which they were fixed and stained with Hoechst to allow for examination of the intracellular growth rates. DMSO treated tachyzoites were able to replicate and give parasitophorous vacuoles containing 2, 4, 8, or 16 parasites at 24 h post-infection (Table 2.2). Most vacuoles (63%) contained eight parasites at 24 h post-infection, whereas at 36 h the majority (50%) vacuoles contained 32. This indicated a doubling time of approximately 6 h. This is similar to that observed in RHΔHX parasites grown without DMSO (Radke et al., 2001). We therefore concluded that the solvent of choice had little effect on intracellular growth rates and proceeded to see the effects of Group A compounds.

Pyrimethamine, securinine and Group A compounds had an inhibitory effect on intracellular growth as can be seen by the decrease in the average vacuole size of these compounds when compared to DMSO treatment (Table 2.2). Compounds 1 and 8 suppressed intracellular growth the most, with the majority of vacuoles containing only two parasites at 24 h. At 36 h, the majority of vacuoles contained eight parasites for all compounds tested with the exception of pyrimethamine, a difference of two replicative cycles from the DMSO control. Although all compounds showed a decrease in average vacuole size, some were unable to decrease growth rate (as shown by doubling time) of the
parasites. Securinine and compound 8 proved to be the most effective, increasing the doubling time by twofold, whereas compound 3 and pyrimethamine only moderately affected the growth rate. Interestingly, compound 1 showed little effect on doubling time perhaps indicating that its anti-Toxoplasma effects rely primarily on an earlier step in the lytic cycle, possibly influencing host cell invasion.

Table 2.2 Inhibition of T. gondii growth by securinine and pyrrolidine derivatives.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time (h)</th>
<th>Number of parasites per vacuole (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Average vacuole size</th>
<th>Doubling time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>DMSO</td>
<td>24</td>
<td>5</td>
<td>24</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>2</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Securinine</td>
<td>24</td>
<td>31</td>
<td>53</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>13</td>
<td>27</td>
<td>37</td>
</tr>
<tr>
<td>Compound 1</td>
<td>24</td>
<td>55</td>
<td>43</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>11</td>
<td>20</td>
<td>39</td>
</tr>
<tr>
<td>Compound 3</td>
<td>24</td>
<td>37</td>
<td>56</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>13</td>
<td>24</td>
<td>38</td>
</tr>
<tr>
<td>Compound 8</td>
<td>24</td>
<td>51</td>
<td>48</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>17</td>
<td>31</td>
<td>46</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>24</td>
<td>23</td>
<td>56</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>2</td>
<td>11</td>
<td>34</td>
</tr>
</tbody>
</table>

<sup>a</sup> Averages of three trials.

We therefore examined whether extracellular tachyzoites pretreated with either DMSO, securinine, compound 1 or pyrimethamine would impair invasion of host cells. An equal number of extracellular tachyzoites were incubated in parasite culture media in the presence of selected compounds at their respective IC<sub>50</sub> for 1 h, and then used to infect HFF monolayers. The number of parasitophorous vacuoles per microscopic field of view was determined in order to give an indication of the parasite’s ability to infect host cells. When treated with compound 1, the average vacuole density (0.35 ± 0.23 vacuoles per field of
view; VPF) decreased drastically by 70% when compared to the DMSO control (1.2 ± 0.28 VPF), indicating that treatment with compound 1 impaired invasion. A moderate decrease of about 40% was also observed in securinine (0.73 ± 0.13 VPF) and pyrimethamine (0.73 ± 0.29 VPF) treatments.

A decrease in the number of vacuoles detected may either be due to a decrease in the ability of the parasite to invade a host cell, or due to a loss of viability of extracellular parasites during the 1 h incubation with the tested compound. We therefore examined the effects of the above compounds on the viability of extracellular tachyzoites. Freshly released extracellular tachyzoites were incubated in culture media in the presence of tested compounds for various durations. Their viability was determined microscopically using propidium iodide staining. Viable parasites are able to exclude the dye, while impaired or dead parasites accumulate the fluorescent dye. As shown in Table 2.3, tachyzoites incubated with the tested compounds show similar viability as those treated with DMSO. We concluded that securinine, compound 1 and pyrimethamine were incapable of eliminating extracellular parasites under the tested conditions. A previous report by Camps and Boothroyd (2001) found that pyrrolidine dithiocarbamate (PDTC), a pyrrolidine-containing compound, was able to selectively kill extracellular *T. gondii* tachyzoites. However, their results suggested that the cidal activity relied on a pro-oxidant pathway, not related to the pyrrolidine ring. Our results support this and suggest that the primary role of the pyrrolidine ring in the extracellular cidal activity of PDTC may simply be to increase its hydrophobic nature, thereby facilitating crossing of the *T. gondii* membrane.
2.3.3 Effect of securinine and pyrrolidine derivatives on parasite differentiation

The rate of replication of intracellular parasites may be indicative of the developmental stage of the parasite. While residing within warm-blooded animals, actively replicating tachyzoites switch into slow growing encysted bradyzoites in response to the immune system attack during disease progression. Since the treatment of infected monolayers with securinine and some Group A compounds slowed the rate of parasite replication, we further investigated whether these compounds can induce *T. gondii* differentiation. Tachyzoites were allowed to invade HFF without any pre-treatment. The infected monolayers were then cultured in the presence of securinine or pyrrolidine derivatives at their respective IC$_{50}$ for 3 days under normal culturing conditions (1% serum, pH 7, 5% CO$_2$). To detect presence of a cyst wall structure, we stained infected monolayers with FITC-labelled *Dolichos biflorus* lectin, which has high affinity to cyst wall glycoproteins. As shown in Figure 3.2A, treatment with DMSO did not cause the formation of encysted parasites (upper panel). Securinine treatment induced the formation of cyst structures (Figure 3.2A, middle panel) similar to those observed by induction under alkali conditions (pH 8.3, atmospheric CO$_2$; Figure 3.2A, lower panel). We thus compared the ability to induce differentiation by securinine to that of alkaline conditions. We detected

Table 2.3 Extracellular tachyzoite viability after compound treatment.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>DMSO</th>
<th>Sec</th>
<th>C1</th>
<th>Py</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>91</td>
<td>82</td>
<td>81</td>
<td>77</td>
</tr>
<tr>
<td>1</td>
<td>92</td>
<td>86</td>
<td>89</td>
<td>82</td>
</tr>
<tr>
<td>2</td>
<td>83</td>
<td>77</td>
<td>88</td>
<td>78</td>
</tr>
<tr>
<td>3</td>
<td>80</td>
<td>78</td>
<td>84</td>
<td>82</td>
</tr>
<tr>
<td>24</td>
<td>35</td>
<td>37</td>
<td>34</td>
<td>30</td>
</tr>
</tbody>
</table>
that approximately the same amount of RHΔHX strain parasites can be induced to form
cysted bradyzoites under both conditions (Figure 3.2B). Interestingly, none of the Group
A compounds tested, including compound 8, which greatly decreased parasite doubling
time, induced the formation of encysted parasites under tested conditions.
Figure 2.2 Securinine causes bradyzoite differentiation *in vitro*.

RHΔHX tachyzoites were incubated for 3 days with selected compounds or in alkaline media. They were then stained with Hoechst (Hz) and with a cyst-specific FITC-conjugated lectin (Dol-FITC) and microscopically examined for bradyzoite formation. (A) Visual comparison of tachyzoites treated with DMSO, securinine or alkaline media for 3 days. Scale bar equals 10 μm. (B) Differentiation rates of RHΔHX parasites incubated for 3 days in selected compounds. Sec, securinine; C1, compound 1; C3, compound 3; C8, compound 8; Py, pyrimethamine.
2.4 Conclusion

We have shown that securinine, a widely used alkaloid in traditional Chinese medicines, is useful as an anti-Toxoplasma agent that induces bradyzoite formation in vitro. Interestingly, Rana et al. (2010) have recently shown that securinine is also able to selectivity kill p53 deficient colon cancer cells. As an agent able to act upon the CNS (Raj and Luczkiewicz, 2008), securinine may have potential in cancer patients who have been previously infected with T. gondii. Upon immune suppression from chemotherapy treatment, bradyzoites residing in the CNS may convert to tachyzoites, leading to toxoplasmic encephalitis (Montoya and Liesenfeld, 2004). The addition of securinine to the chemotherapy mix may assist in both fighting the cancer and maintaining T. gondii in the dormant bradyzoite state.

In addition to securinine, we also examined 10 synthetic precursors from the synthesis of (−)-securinine. Although these pyrrolidine derivatives show varying potential as anti-Toxoplasma agents, they do offer important information in the development of future anti-toxoplasmosis compounds. Their activity seems to correlate best to their hydrophobicity. However, Group A compounds inhibit T. gondii proliferation at different steps of lytic cycle. Compound 1 reduces T. gondii proliferation by limiting its invasion of host cells. Compounds 3 and 8 slow down the intracellular growth rate of T. gondii without inducing bradyzoite differentiation. These compounds show promise as a starting point from which to work forward in the development of novel invasion and growth inhibitors of T. gondii.
2.5 Works cited


CHAPTER III – Sustained translational repression of lactate dehydrogenase 1 in 
*Toxoplasma gondii* bradyzoites is conferred by a small regulatory RNA hairpin

3.1 Introduction

*Toxoplasma gondii* is an obligate intracellular parasite of the phylum Apicomplexa. It infects warm blooded animals with world-wide distribution and is estimated to infect up to a third of people (Pappas *et al.*, 2009; Lafferty, 2006). In intermediate hosts, *T. gondii* exists as one of two haploid stages, the rapidly growing tachyzoite or the dormant bradyzoite. Tachyzoites are generally eliminated by the host’s immune system or drug therapy. Under stressful conditions caused by the induction of the immune response, tachyzoites convert into encysted bradyzoites (Skariah *et al.*, 2010). The presence of encysted bradyzoites is responsible for a chronic infection and is generally non-responsive to the immune system and drug treatments (Moreno and Li, 2008). If a chronically infected host becomes immunocompromised, bradyzoites can reconvert to tachyzoites and cause serious illness (Montoya and Liesenfeld, 2004).

Gene expression is highly regulated throughout the different stages of the *Toxoplasma* life cycle. Determining the mechanisms by which genes are differentially regulated between tachyzoites and bradyzoites will aid in gaining a better understanding of the conversion process. Previous work on transcriptional regulation in *Toxoplasma* has shown that a variety of genes are controlled through stage-specific promoters (Saksouk *et al.*, 2005; Behnke *et al.*, 2008). Research aimed at understanding post-transcriptional regulation has suggested that controlling the translational competency of transcripts is equally important for regulating gene expression, particularly in bradyzoites. The mechanisms controlling translational competency under stressful conditions are crucial for
modulating the survival of parasites within the host and the prognosis of toxoplasmosis (Konrad et al., 2013).

Previous studies have demonstrated that a global translational repression (TR) is induced in Toxoplasma in response to stressful conditions. These conditions include being in the extracellular environment and being subjected to environmental changes that induce differentiation into bradyzoites, examples of which include amino acid starvation and accumulation of unfolded proteins (Joyce et al., 2010; Narasimhan et al., 2008; Konrad et al., 2013; Joyce et al., 2013). The global TR also persists throughout the bradyzoite stage and has been thought to aid in the preferential translation of stress-response proteins (Narasimhan et al., 2008; Joyce et al., 2013; Sullivan et al., 2004). Regulation of the global TR response in Toxoplasma relies on the phosphorylation of eukaryotic initiation factor 2 (eIF2) by eIF2 kinases A-D (Narasimhan et al., 2008; Konrad et al., 2013; Konrad et al., 2011). Phosphorylation by eIF2 kinases also occurs in other organisms under stressful conditions (Sonenberg and Hinnebusch, 2009). Phosphorylated eIF2 has a greater affinity for its nucleotide exchange factor eIF2B, limiting the ability of eIF2B to maintain a pool of functional eIF2 (Sonenberg and Hinnebusch, 2009). This in turn causes a general decrease in protein translation by reducing translational initiation (Sonenberg and Hinnebusch, 2009).

Successful implementation of the global TR response is necessary for the parasite to cope with changes to its environment by allowing the remodeling of its transcriptome. This occurs through two complementary mechanisms. First, a subset of mRNAs is preferentially translated (Joyce et al., 2013). These transcripts correspond primarily to stress-response
proteins and transcription factors which probably induce the transcription of a variety of stress-responsive downstream targets (Joyce et al., 2013). This subset of translationally permissive transcripts has been suggested to be preferentially translated due to the presence of upstream open reading frames within their 5’ untranslated regions (UTRs; Joyce et al., 2013). The second mode by which the transcriptome is remodeled is by removing unneeded transcripts from the translation pool. Many repressed transcripts are probably degraded through standard mRNA decay and turnover (Joyce et al., 2013). Interestingly, in bradyzoites some translationally silent transcripts such as lactate dehydrogenase 1 (LDH1) are maintained (Yang and Parmley, 1997), indicating that another mechanism is acting to selectively withdraw transcripts from the active translation pool and maintain them in a sustained TR throughout stress. This mechanism would allow translationally permissive transcripts access to the limited supply of functional translation machinery (Yamasaki and Anderson, 2008).

It is currently unknown how repressed transcripts are directed for sustained TR in Toxoplasma bradyzoites. The process of storing quiescent transcripts that are needed for the transition between developmental phases is highly conserved across species (Kronja and Orr-Weaver, 2011). In animals for example, transcription is inhibited during late meiosis throughout the initial stages of embryogenesis (Kronja and Orr-Weaver, 2011). Production of new proteins necessary for driving the cell cycle is tightly controlled and relies on the translational activation of previously stored maternal transcripts (Kronja and Orr-Weaver, 2011). In Plasmodium female gametocytes, sustained TR plays a critical role in the maintenance of genes necessary for the transition to zygote. This activity is achieved by the presence of recognizable RNA sequences within these transcripts that are bound by
TR-promoting proteins (Mair et al., 2006; Miao et al., 2013). The widespread conservation of this regulatory system suggests that any transcripts that undergo sustained TR in *Toxoplasma* bradyzoites, such as *LDH1*, are necessary for the reconversion to tachyzoites upon relief of stress.

Other well characterized examples of transcript-specific TR-inducing mechanisms include the targeting of transcripts through the miRNA pathway and through the binding of a variety of regulatory *cis*-acting elements by corresponding RNA binding proteins that act to inhibit translation (Sonenberg and Hinnebusch, 2009). Since there is a large heterogeneity in *cis*-acting elements and RNA binding proteins, they likely function through different mechanisms to achieve a similar result. However, all of these mechanisms share in common the ability to distinguish transcripts through the identification of recognizable *cis*-acting elements, indicating that an RNA-centric view must be taken to study sustained TR. In this study, we sought to determine if *cis*-acting elements could be responsible for the specific targeting of a transcript for sustained TR in *Toxoplasma* bradyzoites.

The gene encoding *LDH1* is unique and suitable for this study because it is one of the only experimentally verified post-transcriptionally regulated genes in bradyzoites (Yang and Parmley, 1997). While the LDH1 protein is present only in tachyzoites, its mRNA is present in both tachyzoites and bradyzoites at similar levels (Yang and Parmley, 1997), strongly suggesting a sustained TR mechanism is at play. Furthermore, it has been shown that this regulation is mediated through the sequences flanking the *LDH1* coding sequence (Yang and Parmley, 1997). We hypothesized that the regulation of *LDH1* is mediated
through a regulatory cis-acting element located in one or both its UTRs that is capable of specifically conferring sustained TR. In this study, we examined the flanking sequences of LDH1 and found a 40 nucleotide element located within its 151 nucleotide 5’UTR. This cis-acting element, termed the K45–85 element, is capable of conferring sustained TR in a heterologous context to transcripts that normally do not exhibit stage-specific regulation. Furthermore, this element requires the formation of a small regulatory RNA hairpin for its activity. These results offer insight into the wider capability of Toxoplasma to selectively distinguish transcripts and target them for sustained TR under stressful conditions.

3.2 Materials and methods

3.2.1 Host cell and parasite culturing conditions

Human foreskin fibroblasts (ATCC: SCRC-1041) were grown to confluency in DMEM medium (Invitrogen, Ontario, Canada) supplemented with 10% cosmic calf serum (Hyclone, Utah, USA) and 0.5x antibiotic-antimycotic (Invitrogen) at 5% CO₂. Confluent monolayers were infected with the type II T. gondii strain PLKΔHX (NIH AIDS program, Division of AIDS, NIAID, cat#2860; Roos et al., 1994; Pfefferkorn and Borotz, 1994) which were grown for 24 h or 5 days under tachyzoite or bradyzoite culturing conditions respectively. Tachyzoites were grown in MEM medium (Invitrogen) supplemented with 1% dialysed fetal bovine serum (Hyclone) and 0.5x antibiotic-antimycotic at 5% CO₂. Bradyzoites were grown at ambient CO₂ levels in alkaline RPMI medium (Invitrogen) supplemented with 5% dialysed fetal bovine serum and buffered with 50 mM HEPES (pH 8.2). Freshly lysed parasites were transformed by electroporation with purified plasmids using a previously established protocol (Roos et al., 1994). Transformed parasites were selected with tachyzoite culture medium containing 20 µg ml⁻¹ mycophenolic acid and 50
µg·ml⁻¹ xanthine. Stable transgenic clonal lines were obtained by isolating single plaques following serial dilution of transformed cultures in 96-well plates.

3.2.2 Plasmid construction

3.2.2.1 Whole UTR constructs

All plasmids created in this study are modified from pTUBmycGFP_HX (Hettmann et al., 2000). This construct, termed pCTRL for this study, contains the α-tubulin TUB8 promoter and 5’UTR upstream of the GFP coding sequence followed by the SAG1 3’UTR (Table 3.1). LDH1 (TGME49_032350) upstream (600 bp containing the promoter and 5’UTR) and downstream (780 bp containing the 3’UTR) flanking regions were obtained by PCR. See Appendix A for a list of oligonucleotides. Constructs containing the flanking regions were created by digesting the relevant PCR fragments and pCTRL with either KpnI and HindIII or PacI and BamHI to form compatible ends and to remove TUB8- or SAG1-related sequences from the vector followed by gel purification and ligation. The construct consisting of GFP flanked by both upstream and downstream LDH1 regions was termed p53L1 because it contains of the 5’ and 3’UTRs of LDH1 (Table 3.1). Similarly in p5L1, GFP is flanked 5’ by LDH1 while in pT3L1 GFP is flanked with LDH1 sequences 3’ while maintaining the TUB8 promoter and 5’UTR (Table 3.1).

The pT5L1 construct was created by digesting p53L1 with EcoRI and PstI to excise the fragment consisting of the LDH1 5’UTR to the distal end of the GFP coding sequence. This was ligated into EcoRI / PstI digested pCTRL. This construct therefore is a fusion of the TUB8 and LDH1 5’UTRs where the TUB8 promoter was used to drive the transcriptional unit (Table 3.1).
Table 3.1 Outline of sequences flanking selected GFP reporter constructs.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>5’UTR</th>
<th>3’UTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTRL</td>
<td>TUB8</td>
<td>TUB8</td>
</tr>
<tr>
<td>P53L1</td>
<td>LDH1</td>
<td>LDH1</td>
</tr>
<tr>
<td>P5L1</td>
<td>LDH1</td>
<td>LDH1</td>
</tr>
<tr>
<td>PT3L1</td>
<td>TUB8</td>
<td>TUB8</td>
</tr>
<tr>
<td>PT5L1</td>
<td>TUB8</td>
<td>TUB8-LDH1</td>
</tr>
<tr>
<td>PT5L1:ΔXX-XXa</td>
<td>TUB8</td>
<td>TUB8-LDH1 (truncated)</td>
</tr>
<tr>
<td>PT5L1:K45-85</td>
<td>TUB8</td>
<td>TUB8-LDH1 (segment)</td>
</tr>
<tr>
<td>PGRA:CTRL</td>
<td>GRA1</td>
<td>GRA1</td>
</tr>
<tr>
<td>PGRA:K45-85</td>
<td>GRA1</td>
<td>GRA1-LDH1 (segment)</td>
</tr>
</tbody>
</table>

a XX represents nucleotides deleted from LDH1 5’UTR.

3.2.2.2 5’ UTR fragment constructs

Deletion mapping of the *LDH1* 5’UTR was done using pT5L1 as a template. Oligonucleotides (Appendix A) were designed to anneal within the *LDH1* 5’UTR back to back (omitting the segment to be deleted) and to introduce an *AfeI* restriction site at the deletion site for screening purposes. PCR amplification was performed as per the manufacturer’s instructions (Phusion High Fidelity, Thermo Scientific, Ontario, Canada) followed by phosphorylation and ligation of PCR products.

Constructs designed to test for the effectiveness of selected 5’UTR *cis*-acting elements were termed pT5L1:K106–151 and pT5L1:K45–85 because they keep the fragment consisting of nucleotides 106-151 or 45-85 and 133-151 respectively and were made from the pT5L1 background (Table 3.1). Briefly, pT5L1:K106–151 was made by deleting nucleotides 2-105 of the *LDH1* 5’UTR in pT5L1 while pT5L1:K45–85 was made by deleting nucleotides 86-132 from pT5L1:Δ2-44 using the process described above. See Appendix A for oligonucleotide combinations.
Constructs where the *TUB8* promoter and 5’UTR were replaced with corresponding *GRA1* (TGME49_070250) fragments were termed GRA:K45–85 and GRA:CTRL where K45–85 and CTRL correspond to above acronyms (Table 3.1). Briefly, 610 bp of the upstream region of *GRA1* was selectively amplified from PLKΔHX genomic DNA by PCR using oligonucleotides “gra1_upstrm_fw” and “gra1_upstrm_rv” (Appendix A). This region has been shown to be effective for driving expression (Messina *et al.*, 1995). pT5L1:K45–85 and pCTRL vectors and the *GRA1* PCR fragment were digested with *Kpn*I and *Eco*RI to excise *TUB8*-related sequences and generate compatible ends followed by ligation.

3.2.3 pT5L1:K45–85 mutational analysis

The *LDH1*-derived sequences from the pT5L1 5’UTR were assessed for predicted secondary structures using the Mfold webserver located at http://mfold.rna.albany.edu/ (Zuker, 2003). Oligonucleotides were designed back to back against predicted stems in order to introduce mutations that either disrupt or restore predicted hairpins as well as to introduce restriction endonuclease sites to allow for plasmid screening (See Appendix A for oligonucleotide list). Plasmids were created in a similar fashion as described above using pT5L1-K45:85 as a template.

3.2.4 Western blot analysis

One million parasites of freshly released tachyzoites or bradyzoite-infected monolayers were harvested and lysed in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Protein samples were resolved on a 10% SDS/PAGE gel. After transfer to a nitrocellulose membrane, blots were blocked in
5% milk for 1 h and probed with α-LDH1 (1:2000; a gift from Dr. Parmley, Palo Alto, CA, USA), α-BAG1 (1:250; a gift from Dr. Weiss, Albert Einstein College, NY, USA), α-GFP (1:2500; Rockland, PA, USA) or α-TUB (12G10, 1:1000; DSHB, IA, USA). Blots were then probed with horseradish-peroxidase-conjugated α-rabbit (1:20 000), α-mouse (1:10 000) or α-goat (1:10 000) secondary antibodies as appropriate. Chemiluminescent signals were visualized on an Alpha-Imager system. Densitometry measurements were obtained from at least three independent blots using the ImageJ software (version 1.42q). A blot was performed using a serially diluted tachyzoite sample and probed with α-TUB to assess the linear range for densitometric analysis (data not shown). A one tailed Student t test assuming unequal variances was performed using the data analysis package available through Microsoft Excel 2007.

3.2.5 Quantitative RT-PCR

RNA was isolated from freshly released tachyzoites or bradyzoite-infected monolayers using the guanidium isothiocyanate-based method according to the manufacturer’s instructions (BioBasic, Ontario, Canada). Isolated RNA (approximately 15 µg) was resuspended in DEPC-treated H2O and 20 mM ribonucleoside vanadyl complexes (NEB, Ontario, Canada) and treated with 1 unit of DNase for 30 minutes at 37°C. Treated RNA samples were then run on a 1 % agarose gel to ensure integrity and complete genomic DNA digestion. The reverse transcriptase reaction was completed as per manufacturer’s instructions (M-MLV; BioBasic, Ontario, Canada) with the exception of dNTPs being present in the reaction mixture at 2 mM. A negative control reaction excluding reverse transcriptase was also performed (NoRT). Real-time RT-PCR was conducted on the StepOne Plus system (ABI, Ontario, Canada) using SyBR green reagents
(KapaBiosystems, Ontario, Canada) as per manufacturer’s instructions. All tachyzoite and bradyzoite samples were run in technical triplicates and LDH1, GFP, and BAG1 signals were normalized using the Δ(ΔCt) method against GAPDH. NoRT reactions were run in conjunction to ensure lack of carry-over DNA contamination. The complete oligonucleotide list is given in Appendix A. Relative mRNA amounts were determined from three independent biological replicates and a two tailed Student t test assuming unequal variances was performed using the data analysis package available through Microsoft Excel 2007.

3.2.6 Fluorescence microscopy

Human foreskin fibroblasts grown to confluency on coverslips were infected with clonal transgenic parasites and allowed to grow under either tachyzoite or bradyzoite culturing conditions for the allotted time. The infected monolayer was then fixed in 3% paraformaldehyde. After DNA staining with 10 µM Hoechst (Invitrogen), coverslips were mounted on slides and infected monolayers were visualized with a Leica DMIRB inverted microscope. All images were taken with a cooled Q-Imaging CCD camera using the Northern Eclipse software (Image Scanning Technology, Ontario, Canada). Images were processed for publication using Adobe Photoshop software (version 7.0).

3.2.7 Relative GFP fluorescence measurement and analysis

GFP-expressing parasites were measured for their relative fluorescent intensity (RFI), determined by the formula RFI = (Iv/Ib) – 1 where Iv and Ib are the average intensity readings for vacuoles and the background respectively as determined by the Northern Eclipse measurement function. This formula allows for the GFP fluorescent signal to be
expressed as a function of the endogenous background signal, thereby minimizing any interference due to local background fluorescence variations. At least 100 vacuoles in each life stage were measured for each clone. Between three and seven clones were analyzed for each construct with no fewer than 300 counts per life stage per construct. For each clone, the distribution of tachyzoite measurements was normalized to have a mean count of 100% expression while the corresponding bradyzoite counts were expressed as a function of cognate tachyzoite readings. For each construct, the descriptive statistics function available in the Microsoft Excel 2007 Data Analysis pack was used to calculate the population means of tachyzoite and bradyzoite samples ($x_{Tz}$ and $x_{Bz}$, respectively) as well as their variances ($\sigma_{Tz}^2$ and $\sigma_{Bz}^2$, respectively). In order to assess the percent change between tachyzoite and bradyzoite samples, the margin of error of the difference between the two means ($x_{Tz} - x_{Bz}$) was assessed at a confidence interval of 99.9% using a two tailed $z$ test. The standard deviation of this distribution was calculated using the formula $\sigma_{Tz-Bz} = \sqrt{\left(\frac{\sigma_{Tz}^2}{n_{Tz}} + \frac{\sigma_{Bz}^2}{n_{Bz}}\right)}$ where $n_{Tz}$ and $n_{Bz}$ represent the tachyzoite and bradyzoite sample size, respectively. To assess the statistical significance of a decrease of $x_{Bz}$ in relation to $x_{Tz}$, a one tailed $z$ test was performed with $\alpha = 0.001$.

3.3 Results

3.3.1 LDH1 is translationally repressed in bradyzoites

The post-transcriptional regulation of LDH1 is likely performed though a sustained TR mechanism, defined here as the sustained inhibition of translation and the maintenance of the transcript throughout the bradyzoite stage. To address whether the sequences flanking the LDH1 coding sequence were able to confer bona fide TR, we adopted a system in which a green fluorescent protein (GFP) reporter was flanked upstream and downstream
by *LDH1*-derived sequences which encompass its promoter, 5’UTR, and 3’UTR. This transgenic parasite strain was named P53L1 (Table 3.1, Figure 3.1A). We compared the expression profile of this parasite to a control GFP reporter flanked by non-related sequences (CTRL; Table 3.1, Figure 3.1A; Hettmann *et al.*, 2000). A previous study has shown that a CAT reporter flanked upstream by tubulin-related sequences and downstream by *SAG1* did not display stage-specific regulation (Bohne *et al.*, 1997).

The transgenic parasite strains were analysed for their reporter expression in tachyzoites and bradyzoites. While the CTRL showed a small non-statistically significant decrease, GFP protein expression was markedly reduced in bradyzoites to 28% ± 22% that of tachyzoites in P53L1 (Figure 3.1B). The reduction of GFP mirrors the large decrease seen in endogenous LDH1 (94% ± 5%; Figure 3.1B) and is in accordance with observations from Yang and Parmley that a CAT reporter flanked on either side by *LDH1*-derived genomic segments yielded a decrease of 70% signal in bradyzoites (Yang and Parmley, 1997). This may reflect the maximal achievable reduction in reporter expression when flanked by *LDH1*-derived genomic sequences. Notably, although efforts were made to load each lane in the western blot with the same number of parasites, a consistent decrease in tubulin (TUB) signal was observed in bradyzoites (Figure 3.1B). While it could be argued that this may represent an effect of general TR due to eIF2α phosphorylation, the specific LDH1 and GFP decreases seen when normalized to TUB suggest rather that variation in TUB signal is probably due to variation in accurate parasite loading.

The steady state levels of *LDH1* and *GFP* mRNA were also analysed in the parental PLKΔHX and transgenic lines. While PLKΔHX bradyzoites showed a 35% decrease in
LDH1 mRNA, it was not significant (P ≥ 0.01; Figure 3.1C). In the tested reporter constructs, GFP transcripts were maintained in tachyzoites and bradyzoites at similar levels (± 10%; Figure 3.1C). Notably, all bradyzoite samples were shown to express BAG1 mRNA and protein, a marker of the bradyzoite stage (Figure 3.1B,C) thus confirming in vitro stage conversion. Taken together, these results confirm that LDH1 is post-transcriptionally regulated through a sustained TR mechanism.
Figure 3.1 Post-transcriptional regulation of *LDH1* and *GFP* reporter constructs.

(A) Graphical representation of P53L1 and CTRL reporter constructs. (B) Western blot and densitometry analysis of LDH1 and GFP protein expression in tachyzoites and bradyzoites. Relative LDH1 and GFP expression levels as measured by densitometry were normalized against α-TUB levels and converted relative to tachyzoite values. Values were obtained from at least three independent experiments. BAG1 was probed as verification of successful bradyzoite differentiation. (C) Relative quantification of mRNA expression of *LDH1*, *GFP* and *BAG1* in tachyzoites and bradyzoites of selected clones. Expression levels were obtained via the Δ(ΔCt) method and normalized against the internal control, GAPDH. Values were obtained from at least three independent experiments. Bars reflect normalized mean ± standard deviation. Dark bars, tachyzoites; light bars, bradyzoites; asterisk, bradyzoite-specific decrease where P ≤ 0.01.
3.3.2 *LDH1* sustained translational repression is mediated through its 5’UTR

To assess whether the upstream or downstream flanking region of *LDH1* is responsible for conferring TR, we constructed two additional transgenic strains named P5L1 and PT3L1 (Table 3.1) with the goal of determining which flanking region was responsible for conferring post-transcriptional regulatory effects. These strains, along with P53L1 and CTRL, were evaluated by fluorescent microscopy and their relative GFP expression was quantified as a function of normalized GFP fluorescent signal (Figure 3.2A, B). In both CTRL and PT3L1 parasites, a similar bradyzoite-specific increase in GFP fluorescent intensity was observed (19% ± 10% and 19% ± 9%, respectively; *P* = 0.999). Furthermore, *GFP* transcript levels remained unchanged between tachyzoites and bradyzoites (Figure 3.2C). This indicated that neither the 3’UTRs of *LDH1* nor *SAG1* contribute any regulatory effects to their transcript and are unlikely to contain any *cis*-acting elements either promoting or inhibiting translation.

The apparent bradyzoite-specific increase in GFP fluorescent intensity was unexpected as western blot densitometry showed that GFP protein levels did not increase in bradyzoites (Figure 3.1B). This apparent increase was likely due to the difference in vacuole morphology seen in *Toxoplasma* tachyzoites and bradyzoites. A tighter clustering of individual parasites within bradyzoite vacuoles resulted in a moderately higher fluorescent signal due to the stacking of parasites over one another; this does not occur in tachyzoites (compare tachyzoite and bradyzoite vacuole morphology in Figure 3.2A). This moderate bradyzoite-specific increase of GFP fluorescence was taken into account and used as a baseline of zero repression for all comparison because the morphology of tachyzoite and bradyzoite vacuoles among all the tested clonal strains remained constant. GFP signals
were quantified to allow for a robust statistical analysis among the different strains. The analysis was performed for individual vacuoles within the population, reflecting the heterogeneity of possible gene regulation and growth. Furthermore, this method permits the rapid collection of a large dataset for each clonal line tested (at least three clonal lines per construct were quantified for each parasite strain mentioned throughout this study).

In contrast to CTRL and PT3L1, both P53L1 and P5L1 which are GFP constructs flanked upstream by \emph{LDH1} sequences (Table 3.1) displayed a decrease of 31% ± 6% and 45% ± 4%, respectively, in fluorescent signal \((P = 0.999)\) while mRNA levels did not significantly change between the two stages (Figure 3.2A-C). These data demonstrated that the sustained TR seen in \emph{LDH1} is due to its 5’ flanking sequence. Since TR is a post-transcriptional regulatory process, the activity is likely restricted to the \emph{LDH1} 5’UTR. To ensure the same level of transcriptional control between \emph{TUB8} and \emph{LDH1} 5’ flanking sequences, we generated the PT5L1 construct where the \emph{LDH1} 5’UTR was fused between the \emph{TUB8} 5’UTR and \emph{GFP} coding sequence used in CTRL (Table 3.1, Figure 3.3A). By doing so, it allowed for the control of any regulatory activities that might have been caused by upstream sequences other than the \emph{LDH1} 5’UTR. Our results showed that the \emph{TUB8} promoter is stronger than that of \emph{LDH1} (Figure 3.2A) and therefore, using the \emph{TUB8} promoter to drive GFP expression in PT5L1, had the added benefit of generating parasites with a brighter fluorescent signal, making the parasites more amenable for visualization. Most importantly, by fusing the two 5’UTRs, it allowed for the direct and non-ambiguous determination whether the \emph{LDH1} 5’UTR was able to induce sustained TR. Analysis of GFP fluorescence in PT5L1 revealed a bradyzoite-specific decrease of 53% ± 3% \((P = 0.999)\) while mRNA levels remained stable (Figures 3.2A-C and 3.3A), indicating TR was
occurring in PT5L1. This conclusively demonstrated that the *LDH1 5’UTR* confers a TR-promoting activity to its transcript. Interestingly, these results also showed that this activity was capable of overriding the constitutive expression profile possibly attributable to the *TUB8 5’UTR*. Since this regulation was likely due to the presence of a *cis*-acting element located within the *LDH1 5’UTR*, we therefore attempted to locate and identify a minimal element.
Figure 3.2 *LDH1* 5’UTR is responsible for conferring TR.

(A) Fluorescent images of representative GFP-containing transgenic parasite strains under tachyzoite and bradyzoite conditions. Scale bar represents 5 μm. (B) Relative GFP fluorescence (RFU) of transformed parasites under tachyzoite and bradyzoite conditions. Bars show normalized average readings ± standard error (*N* ≥ 700) obtained from at least three independent clones per construct. (C) Relative quantification of mRNA expression of *GFP* in tachyzoites and bradyzoites of selected clones. Expression levels were obtained via the Δ(ΔCt) method and normalized against the internal control, *GAPDH*. Values were obtained from at least three independent experiments. Bars reflect normalized mean ± standard deviation. Dark bars, tachyzoites; light bars, bradyzoites; asterisk, bradyzoite-specific decrease where *P* ≤ 0.001.
3.3.3 The **LDH1** 5’UTR contains a *cis*-acting element capable of inducing sustained TR

In order to identify and localize minimal and essential *cis*-acting element(s), segments of approximately either 20 nucleotides or 40 nucleotides were deleted in a stepwise fashion along the **LDH1** 5’UTR of the plasmid used to make PT5L1 (Figure 3.3A). This construct was chosen as a template for deletion studies for three reasons. First, as previously mentioned, the transcriptional unit is driven by the **TUB8** promoter which is stronger than the **LDH1** promoter, generating brighter GFP-containing parasites and making them more amenable to fluorescent visualization (compare GFP intensities in Figure 3.2A). Second, in a fusion 5’UTR, the structural and sequence context near the transcriptional start site is unlikely to change while examining the effects of deletions within the **LDH1** portion. Importantly, 5’UTRs are prone to being regulated by RNA structures (Kozak, 1991). For example, highly structured 5’UTRs can inhibit ribosome scanning while even moderate structural elements near the transcriptional start site can hinder the recruitment of the 40S ribosomal subunit (Kozak, 1991). Because altering the nucleotide sequence can cause local changes in secondary structure (Zuker, 2003), using PT5L1 as a template for deletional analysis of the **LDH1** 5’UTR ensures that the **TUB8** portion of the 5’UTR can insulate against these and other potential unintended regulatory consequences. The final advantage of using the **TUB8-LDH1** fusion 5’UTR is that it would allow for the rapid determination of a deletion’s effect on regulation. Maximal repression would generate a fluorescent signal similar to the reduction of 53% seen in full length PT5L1 (Figure 3.2C). In contrast, since the difference between PT5L1 and CTRL is merely the insertion of the **LDH1** 5’UTR, a deletion that caused the loss of its regulatory activity would result in a fluorescent profile more akin to that of CTRL.
Six deletion constructs were created from the parental PT5L1 (Figure 3.3A). These were named according to which LDH1-derived residues were deleted with nucleotide +1 being the transcriptional start site of LDH1 as predicted by the T. gondii genomics database (http://toxoDB.org version 6.0). Deletion of nucleotides 2-24 maintained a moderate bradyzoite-specific decrease in GFP fluorescence of 21% ± 10% (P = 0.999; Figure 3.3B). In contrast, deletion of nucleotides 26-65 and 47-65 disrupted TR, as seen by their relative GFP fluorescent profile resembling that of CTRL. Elimination of residues 66-105 also moderately alleviated TR (20% ± 10%, P = 0.999; Figure 3.3B). Taken together, this suggests that deletion of residues 26-65 cause a loss of TR, with nucleotides 47-65 constituting a possible minimal cis-acting element. Notably, the abrogation of regions flanking this element (i.e. Δ2-24 and Δ66-105) appeared to reduce its efficacy in conferring post-transcriptional regulation. Upon elimination of nucleotides 86-105, bradyzoites expressed GFP at approximately 43% ± 6 % (P = 0.999) the amount observed in tachyzoites (Figure 3.3B). This is similar to the level seen in the parental PT5L1, strongly suggesting that these residues do not contribute to any post-transcriptional regulatory effect in the LDH1 5’UTR. In contrast, a 5’UTR lacking residues 106-132 displayed a loss of translational repression, suggesting the presence of a second cis-acting element within the LDH1 5’UTR. The terminal nucleotides (133-151) of the LDH1 5’UTR were not analysed through deletion so as not to alter the nucleotide context surrounding the Kozak sequence because this may have impacted translation in an unpredictable manner. Importantly, when relative GFP transcript amounts were evaluated for each of the above mentioned deletion constructs, no significant change in mRNA abundance was seen between tachyzoites and bradyzoites (P ≥ 0.01; Figure 3.3C).
Figure 3.3 Deletion analysis of the \textit{LDH1} 5\textquoteleft{}UTR reveals a minimal and essential \textit{cis}-acting element.

(A) Graphical representation of deletions along the \textit{LDH1} 5\textquoteleft{}UTR of PT5L1. \textit{TUB8} and \textit{GFP} sequences are in purple and green, respectively, while \textit{LDH1} sequences are orange.

(B) Relative GFP fluorescence (RFU) of reporter 5\textquoteleft{}UTR deletion constructs under tachyzoite and bradyzoite conditions. Bars show normalized average readings ± standard error (\(N \geq 300\)) obtained from at least three clones per construct.

(C) Relative quantification of mRNA expression of \textit{GFP} in tachyzoites and bradyzoites of selected clones. Expression levels were obtained via the \(\Delta(\Delta\text{Ct})\) method and normalized against the internal control, \textit{GAPDH}. Values were obtained from at least three independent experiments. Bars reflect normalized mean ± standard deviation. Dark bars, tachyzoites; light bars, bradyzoites; asterisk, bradyzoite-specific decrease where \(P \leq 0.001\).
To validate these elements as self-contained *bona fide* cis-acting elements that are capable of conferring TR, we constructed plasmids that retained these elements in the CTRL background and named the transgenic parasites lines PT5L1:K106–151 and PT5L1:K45–85 (Table 3.1, Figure 3.4A). In both of these constructs we also included nucleotides 133-151 so as not to interfere with the nucleotide context surrounding the Kozak sequence as outlined above. In the latter construct, although the minimal cis-acting element was outlined by 47-65 through deletional analysis, we opted to use residues 45-85 because the K45–85 region of the *LDH1* 5’UTR was predicted to have a modest amount of secondary structure (discussed further below; Figures 3.4A and 3.5A). The transformed parasites were then analysed for relative GFP expression in bradyzoites. The putative element contained within nucleotides 106-151 did not cause bradyzoite-specific downregulation of GFP expression (Figure 3.4B), thus indicating that this region alone is not sufficient to confer TR. This may have been because the element was not in the proper context, could not adopt the proper conformation, or it may need to work cooperatively with other regions of the *LDH1* 5’UTR in order to function. Since this element was not self-sufficient it was not further investigated. As previously mentioned, the terminal nucleotides of the *LDH1* 5’UTR (from 133-151) were not screened by deletional analysis in the above PT5L1 model (Figure 3.3A). However, by including these nucleotides with the putative 106-132 element, we inferred that this region likely does not contribute regulatory effects. When assessed for GFP expression, PT5L1:K45–85 displayed a 41% ± 6% (*P = 0.999*) bradyzoite-specific reduction in fluorescence which was similar to that seen in the parental PT5L1 (Figures 3.2B and 3.4B) while no significant change in *GFP* mRNA was seen (*P ≥ 0.01*; Figure 3.4C). Taken together, this strongly suggested that these
residues, termed the K45–85 element, constitute a self-contained *cis*-acting element capable of causing TR in the CTRL background.

To verify the ability of the K45–85 element to induce sustained TR in exogenous transcripts, another constitutively expressed gene, *GRA1*, was introduced into the upstream flanking region. The *GRA1* gene is highly expressed in both tachyzoites and bradyzoites and a previous report has shown that this region is sufficient to constitutively express a protein at high abundance in *T. gondii* (Messina *et al.*, 1995). A pair of constructs were created replacing *TUB8* sequences by the promoter and 5’UTR from *GRA1* and named PGRA:CTRL and PGRA:K45–85 (Table 3.1). A decrease of 29% ± 6% (*P* = 0.999) in bradyzoite fluorescence was seen by PGRA:K45–85 while no significant difference was seen in PGRA:CTRL (*P* ≥ 0.01; Figure 3.4D). Also, *GFP* mRNA levels remained statistically unchanged between tachyzoites and bradyzoites (Figure 3.4E). These findings confirm that the K45–85 element is a functional TR-inducing *cis*-acting element. Furthermore, inclusion of this element overrides the constitutive expression seen when either the *TUB8* or *GRA1* upstream flanking regions alone are present, suggesting that the strong sustained TR seen is signaled through the K45–85 element.
Figure 3.4 Nucleotides 45-85 can induce TR in an exogenous system.

(A) *LDH1* 5’UTR sequence. Sequences kept for PT5L1:K106–151 are underlined, while sequences included in PT5L1:K45–85 are capitalized. (B), (D) Relative GFP fluorescence (RFU) of selected *LDH1* 5’UTR elements under tachyzoite and bradyzoite conditions. All bars show normalized average readings ± standard error (*N* ≥ 300) obtained from at least three clones per construct. (C), (E) Relative quantification of mRNA expression of *GFP* in tachyzoites and bradyzoites of selected clones. Expression levels were obtained via the Δ(ΔCt) method and normalized against the internal control, *GAPDH*. Values were obtained from at least three independent experiments. Bars reflect normalized mean ± standard deviation. Dark bars, tachyzoites; light bars, bradyzoites; asterisk, bradyzoite-specific decrease where *P* ≤ 0.001.
3.3.4 Mutational analysis of K45–85 element reveals a small regulatory RNA hairpin

Since the K45–85 element is a strong regulatory element, we hypothesized it would be highly conserved in *Toxoplasma*. In order to probe the transcriptome for similarly regulated transcripts, we generated a MEME motif (Bailey *et al.*, 2009) using the *LDH1* 5’UTR variants selected so as to account for conservation of loss of TR activity (Figure 3.3B). This motif was then used for a MAST search (Bailey *et al.*, 2009) against the annotated non-redundant UTRs of the *Toxoplasma* type II transcriptome as generated by toxodb.org using EST-based evidence. This method identified 12 genes (excluding *LDH1*) with unacceptably high expected values (data not shown). It was therefore concluded that this *cis*-acting element was either not detectable in the transcriptome without further refinement for other TR genes or it is unlikely to be conserved at the sequence level.

It had been noted during the analysis of the *LDH1* 5’UTR that deletions targeting areas immediately flanking the minimal *cis*-acting element located between nucleotides 47-65 caused a moderate loss of TR (Figure 3.3B). Importantly, since RNA folding is in part a thermodynamic process (Zuker, 2003), alterations in the sequence composition at one location can cause changes in structure that influence the folding of adjacent regions. It was therefore hypothesized that observations showing that deletion of sequences located immediately upstream or downstream of the minimal *cis*-acting element caused a moderate impairment of TR may have indicated that its activity relied on the adoption of a particular structural conformation (Figure 3.3B). We therefore examined whether the K45–85
element owed its strong TR-inducing activity to the formation of a specific regulatory secondary structure.

The secondary structures that mRNAs can adopt are varied and have been implicated in a variety of regulatory functions. The formation of strong structures to the order of $-50 \text{ kcal}\cdot\text{mol}^{-1}$ in the 5’UTR can inhibit scanning of the 40S ribosomal subunit (Kozak, 1991) which could explain the ability of the K45–85 element to confer sustained TR in the tested fusion 5’UTRs. To see if such a mechanism could be responsible, the potential for the $LDH1$ 5’UTR (151 nucleotides + 44 nucleotides of coding sequence downstream) to form inhibitory structures was assessed using the RNA secondary structure prediction software Mfold (Zuker, 2003). We selected the predicted structure with the lowest free energy as it should represent the most stable structure (Figure 3.5A). While the level of secondary structure obtained appeared to be insufficient to inhibit ribosomal scanning, the presence of two RNA hairpins was found in the location corresponding to the K45–85 element (Figure 3.4A). Notably these two hairpins, termed Hp1 and Hp2 respectively, are maintained when analysing the K45–85 element in the context of PT5L1:K45–85 (Figure 3.5B). Interestingly, it has previously been established that RNA hairpins can serve various regulatory functions (Svoboda and Di Cara, 2006). We therefore examined whether the formation of these hairpins was important for mRNA regulation and further tested the ability of these predicted structural elements to regulate GFP expression in bradyzoites.

To assess the effects of Hp1 formation on TR, destabilizing mutations within the PT5L1:K45–85 construct were designed to disrupt base pairing between the stems of Hp1 (see Materials and Methods for details). Parasites containing this construct were termed
Hp1D (Figure 3.5C). Corresponding rescue mutations were designed to restore base pairing of the stems and this construct was termed Hp1R (Figure 3.5C). In selecting mutations, care was used to maintain a predicted C:A base pair between residues 55 and 66 (see arrowhead, Figure 3.5A,B) and to ensure the formation of a hairpin with a similar calculated free energy, and therefore similar stability, as the parental PT5L1:K45–85. Disruption of Hp1 caused a loss of TR while the restorative mutations (Hp1R) partially rescued TR as evidenced by a bradyzoite-specific decrease of 25% ± 7% in GFP fluorescence ($P = 0.999$; Figure 3.5D). Furthermore, no significant changes were observed in GFP transcript levels ($P \geq 0.01$; Figure 3.5E). Taken together, these data suggest that a hairpin involving the base pairing of nucleotides 53-57 with 64-68 is crucial for regulation. Previous reports have suggested that non Watson-Crick base pairing may serve an essential role for the recognition of certain RNA hairpins (Hermann and Westhof, 1999), raising the possibility that the predicted C:A pair may serve as a defining feature of this regulatory element. To test this possibility, the Hp1R-UA construct was made in which the pair in question was mutated to form a standard Watson-Crick U:A base pair (Figure 3.5C). Interestingly, the mutation caused a complete loss of bradyzoite-specific GFP regulation and led to a profile akin to that seen in the original CTRL construct with an increase of 17% ± 8% ($P = 0.999$; Figures 3.2B and 3.5D) while mRNA abundance was maintained at similar levels in tachyzoites and bradyzoites ($P \geq 0.01$; Figure 3.5E).

The potential involvement of Hp2 was also investigated. Parasites with disruptive and restorative mutations targeted against Hp2, termed Hp2D and Hp2R respectively, were generated as described above (Figure 3.5C). Both types of mutations designed against Hp2 resulted in similar bradyzoite-specific decreases in GFP expression (26% ± 5% and 24% ±
5% respectively, \( P = 0.999 \); Figure 3.5D) while no change in mRNA abundance was seen (Figure 3.5E), suggesting that mutations to the distal part of the K45–85 element cause only a partial loss of TR. Since no difference was observed between Hp2D and Hp2R, it seems likely that only the formation of Hp1 is necessary for inducing TR.
Figure 3.5 Mutational analysis of K45–85 element reveals essential structural component.

(A) Secondary structure of LDH1 5’UTR was predicted using the MFOLD webserver (Zuker, 2003). Forty-four nucleotides of the plasmid-derived sequence downstream of the LDH1 5’UTR were included in the predicted fold. (B) Predicted secondary structure of K45–85 element in PT5L1:K45–85. Two predicted hairpin structures are noted (Hp1 and Hp2). (C) Multiple sequence alignment of PT5L1:K45–85 and mutated constructs. The shaded area represents the K45–85 element. Sequences predicted to base pair in Hp1 and Hp2 are underlined and mutated residues are capitalized. (D) Relative GFP fluorescence (RFU) of hairpin disrupting and restorative constructs under tachyzoite and bradyzoite conditions. All bars show normalized average readings ± standard error (N ≥ 300) obtained from at least three clones per construct. (E) Relative quantification of mRNA expression of GFP in tachyzoites and bradyzoites of selected clones. Expression levels were obtained via the Δ(ΔCt) method and normalized against the internal control, GAPDH. Values were obtained from at least three independent experiments. Bars reflect normalized mean ± standard deviation. Dark bars, tachyzoites; light bars, bradyzoites; asterisk, bradyzoite-specific decrease where P ≤ 0.001.
3.4 Discussion

3.4.1 Structural requirements of Hp1 suggest a recognition site for a \textit{trans}-acting factor that is capable of targeting the \textit{LDH1} transcript for sustained TR.

The predicted secondary structure of the \textit{LDH1} 5’UTR and mutational analysis of Hp1 suggested that an essential regulatory RNA hairpin is formed from position 53-68. Furthermore, base pairing between residues 53-57 with 64-68 as well as a non-canonical C:A pairing between residues 55 and 66 appear essential to its activity (Figure 3.5). Previous studies have shown that non-canonical base pairing between residues within the stem of RNA hairpins may serve as important recognition points for ribonucleoprotein assembly (Hermann and Westhof, 1999). As RNA hairpins have been known to serve as potential nucleation sites for the assembly of regulatory ribonucleoproteins (Svoboda and Di Cara, 2006), it is possible that Hp1 serves this function in \textit{LDH1} to allow the binding of an unidentified TR-promoting \textit{trans}-acting factor. Upon the binding of such a factor, the transcript could be removed from the active translational pool. This is a common theme often repeated in the literature. For example, in mammals the post-transcriptional regulation of ferritin and other genes involved in iron metabolism relies on a 5’UTR-located conserved RNA hairpin termed the iron response element. In periods of iron depletion, the iron response element is bound by the iron response protein, causing its withdrawal from the translational pool (Muckenthaler \textit{et al.}, 1998).

The proposed unknown \textit{trans}-acting factor involved in \textit{LDH1} TR could act similarly where it may either be responsible for, or contribute to, the recruitment of other factors necessary for protecting the transcript from degradation during the bradyzoite stage where it could be stored until translational reactivation in tachyzoites. Such transcript
maintenance could be mediated through its recruitment to areas of cytoplasmic storage known as stress granules (SGs). In other organisms, SGs are well characterized cytoplasmic bodies that form under the same stressful conditions that cause eIF2 phosphorylation (Yamasaki and Anderson, 2008). They selectively store transcripts throughout stress and it has been suggested that this serves to withdraw them from the limited functional translational machinery, thereby giving preference to the translation of stress-response transcripts (Yamasaki and Anderson, 2008; Kedersha and Anderson, 2002). Furthermore, these structures have been implicated in the maintenance of important mRNAs during stressful conditions and allow for their eventual translational reactivation upon relief of stress (Hooper and Hilliker, 2013). Interestingly, a previous report has shown that mRNA-containing granules with the properties of SGs appear in Toxoplasma under stressful conditions (Lirussi and Matrajt, 2011), suggesting a possible link between global TR and the maintenance of specific transcripts throughout stressful conditions. It is possible to speculate that LDH1, as transcript that undergoes sustained TR in bradyzoites, could be one transcript that is selectively recruited to SGs under stress.

The binding of specific mRNAs by RNA binding proteins that are necessary for maintaining them throughout sustained TR is a recurrent theme. For example, under stressful conditions that cause the inactivation of mTOR in mammals, 5’ terminal oligopyrimidine tract-containing mRNAs are bound by the translational repressors TIA1 and TIAR. These transcripts are withdrawn from active translation and stored in SGs (Damgaard and Lykke-Anderson, 2011). In Plasmodium the RNA binding proteins DOZI and PUF2 have been shown to selectively bind transcripts through a sequence-based cis-acting element and are essential for their maintenance throughout sustained TR (Mair et
al., 2006; Miao et al., 2013; Braks et al., 2008). Notably, the Toxoplasma genome is predicted to contain homologues to these and other regulatory RNA binding proteins. In Toxoplasma, ALBA1 and ALBA2 proteins are capable of binding mRNAs as well as translation regulatory machinery and they localize to SGs under stressful conditions (Gissot et al., 2013).

More recently, Liu and colleagues identified a Toxoplasma homologue of the translational repressor PUF1 that is upregulated and localized to cytoplasmic granules in bradyzoites (Liu et al., 2014). PUF1 binds transcripts through the Puf-binding element, a UUGUN3A consensuse sequence, which is strikingly similar to the sequence starting within the terminal loop of Hp1 composed of UUGUN3A where the A is predicted to form a non-canonical pair necessary for its TR-inducing activity (Figure 5). It is therefore very tempting to speculate on the involvement of PUF1 in the regulation of LDH1 translation in bradyzoites. Identification of trans-acting factors involved in the sustained TR of LDH1 may yield insight into how other similarly regulated transcripts are maintained in Toxoplasma bradyzoites.

A large scale conservation of the K45–85 element amongst the Toxoplasma transcriptome was not found. This is likely because its activity relies on the adoption of certain conformational characteristics and not on a strict sequence-based conservation. Recently Joyce and colleagues have published microarray data listing genes that undergo TR upon induction of the unfolded protein response (Joyce et al., 2013). Unfortunately, it appears they were unable to identify unifying cis-acting elements present within genes targeted for sustained TR. This may illustrate either the need for a comparative RNA
structure approach or a large variety in potential TR-inducing cis-acting elements. Intriguingly, a survey of the literature studying post-transcriptional gene regulation in Toxoplasma points to a rich abundance of cis-acting elements in its transcriptome. For example, a report on the apicoplast-targeted translational elongation factor G has suggested that its 3’UTR contributes to promoting its translation, suggesting the presence of an undefined cis-acting element within (Payne et al., 2011). Also the translation of ALBA2 requires a putative element located in its 3’UTR (Gissot et al., 2013). Limited reports, coupled with the discovery of miRNA and its differential regulation in Toxoplasma (Braun et al., 2010), strongly suggest that transcript-specific post-transcriptional gene regulation is important for modulating gene expression through the use of cis-acting elements. This may be reflective of the need for multiple transcript-specific regulatory mechanisms to adequately control gene expression in Toxoplasma.

3.4.2 Sustained TR of LDH1 suggests that its regulation is important for bradyzoite and tachyzoite interconversion

Carbohydrate metabolism in bradyzoites is radically different than in tachyzoites. Bradyzoites lack the ability to complete the tricarboxylic acid cycle and their mitochondrion is non-functional (Denton et al., 1996; Toursel et al., 2000) suggesting that they operate under functionally anaerobic conditions. Since bradyzoites are unable to complete cellular respiration, glycolysis is used as a major source of energy (Denton et al., 1996). In the final step of glycolysis under anaerobic conditions, lactate dehydrogenase catalyses the conversion of pyruvate to lactate and regenerates NAD\(^+\) from NADH. In bradyzoites, this reaction is catalysed by LDH2 which is transcriptionally upregulated to perform this task (Behnke et al., 2008; Yang and Parmley, 1997). The production of lactate
is much more active in bradyzoites than in tachyzoites, suggesting that a higher throughput of this process is necessary for the parasite to meet its energy requirements (Denton et al., 1996). LDH2 functions efficiently in the presence of the large amounts of pyruvate assumed to be generated in bradyzoites (Dando et al., 2001). This characteristic is thought to be essential for the regeneration of NAD$^+$ and the continual drive of glycolysis throughout anaerobic conditions. In contrast, the function of LDH1 is inhibited by large doses of pyruvate, suggesting that it is not adapted to the bradyzoite environment (Dando et al., 2001). Furthermore, two reports have shown that alterations in the expression profiles and levels of the LDH isoforms can influence the parasite’s ability to differentiate, suggesting that precise regulation of LDH isoforms must be tightly controlled (Al-Anouti et al., 2009; Liwak and Ananvoranich, 2009).

In tachyzoites LDH2 is transcriptionally silenced while LDH1 protein expression is made possible. Perhaps surprisingly, although the tricarboxylic acid cycle is functional, it has been shown that the conversion of pyruvate to lactate still occurs at substantial levels in tachyzoites (Denton et al., 1996). As much as 20% of the glucose taken in by tachyzoites may be converted to lactate (Denton et al., 1996), highlighting the need for a functional LDH isoform in this stage. As such a large contributor of energy to the parasite, it could be hypothesized that production of functional LDH1 protein may be an early development in the transition of bradyzoite to tachyzoite. Upon the relief of stress, the LDH1 transcript would be released from sustained TR and made translationally competent, allowing for Toxoplasma to rapidly respond by compensating for the loss of LDH2 through immediate production of LDH1. Such a mechanism would be faster and more energetically favourable than newly transcribing the LDH1 gene upon conversion to tachyzoites.
3.5 Conclusion

In this study, we identified a small structural cis-acting element that resides within the LDH1 5’UTR. To our knowledge, this is the first report offering the experimental characterization of a specific cis-acting element that contributes to post-transcriptional gene regulation in Toxoplasma. We show that this element selectively targets the LDH1 transcript for sustained TR and can confer stage-specific expression to an otherwise constitutively expressed gene in a heterologous context. This activity requires the adoption of an RNA hairpin structure which may be indicative of an RNA protein binding site. The selectivity conferred by the K45–85 element strongly suggests that the LDH1 transcript which is destined for sustained TR is recognized by as of yet unidentified trans-acting factor(s) in Toxoplasma bradyzoites. We further propose that upon identification by said factors, the transcript is withdrawn from the pool of actively translated mRNAs and sent for maintenance and storage. Upon the relief of stress, it would be translationally reactivated in order to aid in the reconversion into tachyzoites. By studying similarly regulated transcripts, it may be able to illuminate pathways, metabolic or otherwise, that need to be rapidly reactivated upon the relief of stress for the reconversion to tachyzoites.
3.6 Works Cited


CHAPTER IV – The use of RNA aptamer technologies for the affinity purification of 
LDH1-interacting partners and examination of short- and long-term features of stress-
induced translational repression in *Toxoplasma gondii*

4.1 Introduction

The world’s most successful parasite, the obligate intracellular pathogen *Toxoplasma gondii*, infects warm-blooded animals the world over, including up to an estimated one third of the human population (Turner *et al.*, 2013; Montoya and Liesenfeld, 2004). Key to its survival and widespread distribution is its ability to react to the many hostile environments it encounters throughout its sexual and asexual lifecycles (Skariah *et al.*, 2010). After completing its sexual reproduction in the intestine of the feline, it must survive in the outdoor environment for months or years until being ingested by a warm-blooded host (Dubey, 1998; Torrey and Yolken, 2013). Once ingested, it must survive the harsh extracellular environment it encounters after destroying a previously infected host cell until it colonizes another (Konrad *et al.*, 2011; Black and Boothroyd, 2000). Once infection is established in an intermediate host, it must survive the anti-microbial effects of interferon γ produced by the innate immune system (Suzuki *et al.*, 1988; Schroder *et al.*, 2004). This is accomplished by converting from the rapidly growing and destructive tachyzoite stage to the non-replicating and immunologically protected bradyzoite stage which is responsible for maintaining chronic infection for the life of the host (Skariah *et al.*, 2010).

Similar to other eukaryotes, when subjected to stressful stimuli, *Toxoplasma* initiates a robust general translational repression mediated through the phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α; Sullivan *et al.*, 2004). Phosphorylation of the Ser71 regulatory residue causes eIF2 to have an increased affinity for its nucleotide
exchange factor, decreasing its turnover rate and inhibiting cap-dependent translation (Sullivan et al., 2004; Sonenberg and Hinnebusch, 2009). eIF2α phosphorylation is performed by one of four *T. gondii* eIF2α kinases (TgIF2K)s, each of which are activated by different signals (Narasimhan et al., 2008). TgI2K-A is activated in response to the accumulation of misfolded proteins in its endoplasmic reticulum, TgIF2K-B is induced by oxidative stress and other cytoplasmic stresses, while TgIF2K-C and TgIF2-D both react to amino acid starvation, with the former selectively acting within the intracellular environment and the later exclusively mediating the response when the parasite finds itself in the extracellular milieu (Narasimhan et al., 2008; Konrad et al., 2013a; Konrad et al., 2011; Joyce et al., 2010).

Prolonged eIF2α phosphorylation causes the conversion of tachyzoites into bradyzoites and the translational control demonstrated under stressful conditions has been suggested to be a key part of the conversion mechanism (Konrad et al., 2013b; Joyce et al., 2013). While under eIF2α phosphorylation, a subset of transcripts that encode stress-responsive proteins are preferentially translated while the majority dissociate from polysomes (Joyce et al., 2013). Of this latter group, most appear to be degraded through standard mRNA turnover (Joyce et al., 2013). However, a subset of translationally quiescent transcripts is maintained throughout the prolonged TR response (Yang and Parmley, 1997; Dendouga et al., 2002; Cleary et al., 2005). This suggests a mechanism exists by which they are distinguished from those transcripts subject to degradation and would rely on the use of recognizable *cis*-acting elements.
Previously we have proposed that non-translated transcripts that are maintained throughout the bradyzoite stage are likely recruited to stress granules (SG)s, sites of mRNA storage that are formed in response to stressful environments (Holmes et al., 2014; Anderson and Kedersha, 2008). In well studied eukaryotic organisms, SGs have been found to be formed upon eIF2α phosphorylation and consist of translationally quiescent mRNAs, machinery involved in cap-dependent translational initiation, and RNA-binding proteins that bind specific cis-acting elements (Anderson and Kedersha, 2008). Importantly, SGs have recently been shown to form in both extracellular tachyzoites and intracellular bradyzoites in *Toxoplasma*, demonstrating that this stress-response pathway is conserved in ancient eukaryotes (Lirrusi and Matrajt, 2011; Cherry and Ananvoranich, 2014; Cherry and Ananvoranich, unpublished results).

In our previous work, we used the lactate dehydrogenase 1 (*LDH1*) transcript as a study model to determine how specific transcripts are selected to be maintained through what we termed long-term sustained translational repression. It was determined that *LDH1* harbours a cis-acting element, termed the K45–85 element, in its 5’UTR that is responsible for inhibiting its translation in bradyzoites while allowing the mRNA to be maintained at similar steady state levels to what is seen in tachyzoites (Holmes et al., 2014). The K45–85 element necessitates the formation of a small 16 nucleotide RNA hairpin that is characterized by a central non-canonical C:A base pair. It is well documented that internal non-canonical base pairings and RNA hairpin loops can be important recognizable features that mediate the binding of RNA-binding proteins (Hermann and Westhof, 1999; Leontis and Westhof, 2003). It was therefore hypothesized that upon eIF2α phosphorylation, the
*LDH1* transcript is recruited to SGs through the actions of an unidentified *trans*-acting factor (Holmes *et al.*, 2014).

RNA aptamers are small, self-folding RNA segments that specifically bind a molecule of interest (Mayer, 2009). They have been discovered in naturally-occurring biological systems or engineered from randomized RNA libraries through successive rounds of selective amplification of ligand-binding RNAs (Mayer, 2009). RNA aptamers combined with affinity purification procedures are exceptionally suited to offer an unbiased approach towards identifying the protein complement that binds a known transcript (Srisawat and Engelke, 2001; Slobodin and Gerst, 2010). Since *LDH1* has been demonstrated to be an ideal model of sustained translational repression in bradyzoites (Holmes *et al.*, 2014), in this study the feasibility of using RNA aptamer technologies in combination with the *LDH1* system were examined. Furthermore, to better understand how *T. gondii* manages stress, the *LDH1* system was used to better examine the features of stress-induced translational control.

4.2 Materials and methods

4.2.1 Plasmid construction

All polymerase chain reactions (PCR)s performed to generate plasmids were conducted according to the manufacturer’s instructions (Phusion High Fidelity, Thermo Scientific, Ontario, Canada). The template and primer pairings for each construct are listed in Appendix B.
4.2.1.1 Aptamer constructs

All aptamer-containing constructs generated in this study were based off of the green fluorescent protein (GFP) reporter system flanked upstream by \( LDH1 \)-related and/or tubulin-related sequences and downstream by either \( LDH1 \) or \( SAG1 \) sequences (Holmes et al., 2014). The 44 nucleotide minimal S1 aptamer, which selectively binds streptavidin (Srisawat and Engelke, 2001), was inserted along with an \( EcoRV \) endonuclease restriction site directly downstream of the \( GFP \) stop codon by a PCR approach using back to back primers. After PCR, the products were phosphorylated with T4 polynucleotide kinase according to the manufacturer’s instructions (Promega, Ontario, Canada) and ligated with T4 DNA ligase for 1 h at 37°C (BioBasic, Ontario, Canada). After bacterial transformation, clones were screened for the incorporation of the \( EcoRV \) restriction site. This generated plasmids p53L1-S1 and pT3L1-S1 from p53L1 and pT3L1, which both contain the S1 aptamer and differ from each other only by their use of \( LDH1 \) or \( TUB8 \) upstream fragments, respectively (Table 4.1).

Plasmid p53L1-S1 was used to create pT5L1-S1 and pCTRL-S1. To do so, parental plasmids pT5L1 and pCTRL as well as p53L1-S1 were digested with \( NsiI \) and \( PacI \) and gel purified (Hettmann et al., 2000; Holmes et al., 2014). The \( GFP \)-S1 fragment generated from p53L1-S1 was ligated into the opened and \( GFP \) deficient pT5L1 and pCTRL vectors to generate pT5L1-S1 and pCTRL-S1 respectively. These later plasmids are both driven by a modified tubulin promoter with transcription initiating at the \( TUB8 \) 5’UTR and also have \( SAG1 \) 3’UTRs, similar to the previously established system (Table 4.1; Hettmann et al., 2000; Holmes et al., 2014). However, in this study plasmids pT5L1-S1 and pCTRL-
S1 were not used for parasite expression and instead used for further cloning or for the generation of in vitro synthesized RNA (Appendix B).

Plasmids containing the 68 nucleotide Hepatitis delta virus (HDV) ribozyme were generated in a similar back-to-back strategy as described above in a two-step process. The HDV ribozyme, which is capable of self-cleaving directly 5’ of itself (Mercure et al., 1998), was inserted directly downstream of the S1 aptamer using oligonucleotides that each incorporated a partial modified HDV genomic strand ribozyme (Been, 2006). This version of the HDV ribozyme contains an altered P1 stem and a shortened P4 stem, two changes which have been shown to have negligible impact on its function (Mercure et al., 1998; Ananvoranich et al., 1999). Once ligated, the oligonucleotides generated a SphI restriction digest site for screening purposes and, used in conjunction with pT5L1-S1 or pCTRL-S1 in a PCR reaction yielded pT5L1-S1-HDVΔG58 and pCTRL-S1-HDVΔG58, respectively (Table 4.1). These plasmids encode a catalytically inactive form of the HDV ribozyme (Been and Perrotta, 1995). In the second step, these plasmids were used as templates to create pT5L1-S1-HDV and pCTRL-S1-HDV, which each lack a PacI site for screening and encode a catalytically active ribozyme (Table 4.1).

Plasmids were also designed for use with a different aptamer system derived from the MS2 bacteriophage (Slobodin and Gerst, 2010). The MS2 aptamer, consisting of two 19 nucleotide RNA hairpins separated by a small spacer, selectively binds the MS2 phage coat protein (CP), which in turn can be used for affinity purification (Said et al., 2009; Slobodin and Gerst, 2010). Using a similar back-to-back methodology as described above, one copy of a MS2 hairpin was incorporated into each oligonucleotide so that they
generated plasmids encoding two copies of the MS2 hairpin separated by an XbaI restriction site directly downstream of the GFP stop codon. These plasmids were named pT5L1-MS2 and pCTRL-MS2 (Table 4.1).

4.2.1.2 Dual luciferase constructs

Plasmids incorporating the firefly (FF) or Renilla (RN) luciferase coding sequences were adopted to the previously established LDH1 GFP reporter system (Holmes et al., 2014). The FF luciferase fragment was obtained by gel purification after digesting plasmid p3011FF with NsiI and PstI. Vectors pT5L1 and pCTRL were digested with NsiI and PstI in order to remove the GFP coding sequence, dephosphorylated with calf intestinal phosphatase according to the manufacturer’s instructions to prevent self-ligation (Promega), and gel purified. The appropriate fragments were then ligated and, after transformation, screened for insertion in the correct orientation with EcoRI and PstI. This generated plasmids pT5FF and pT0FF (Table 4.1).

To serve as an internal control for dual luciferase expression assays, a RN-containing plasmid was generated and termed pT0RN (Table 4.1). Briefly, the RN coding sequence was PCR-amplified from pTubRn (Crater et al., 2012), digested with PstI and ligated within an NsiI / PstI digested and dephosphorylated open pT0FF vector following a similar procedure as previously mentioned.
Table 4.1 Names and characteristics of plasmids used in study.

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4.2.1.3 Bacterial expression vector

For use in subsequent affinity purification, a plasmid was generated so as to bacterially express a fusion protein consisting of an N-terminal CP, a central GFP, and a C-terminal streptavidin-binding peptide (SBP). The sequence corresponding to CP-GFP-SBP fusion protein was PCR-amplified from a plasmid provided by Dr. Jeffrey Gerst (Weizmann Institute of Science, Israel). The PCR fragment was digested with BspHI and XhoI and gel purified. A pET28b vector was digested with NcoI and XhoI, gel purified and the ligated with the CP-GFP-SBP fragment. The resulting plasmid, pET_CP-GFP-SBP, was subsequently used to bacterially express C-terminally histidine tagged CP-GFP-SBP.
4.2.2 Tissue culture and parasite transformation

Routine culture of human foreskin fibroblasts (ATCC: SCRC-1041) was conducted using DMEM medium (Invitrogen) supplemented with 10% cosmic calf serum (Hyclone, USA) and 0.5x antibiotic-antimycotic (Invitrogen). Upon reaching confluency at 37°C in a 5% CO₂ atmosphere, fibroblasts were infected with the *Toxoplasma gondii* PLKΔHX strain which lacks functional HXGPRT activity (NIH AIDS program, Division of AIDS, NIAID, cat#2860; Roos *et al.*, 1994; Pfefferkorn and Borotz, 1994). Tachyzoites were grown in MEM medium (Invitrogen) supplemented with 1% dialysed fetal bovine serum (Hyclone) and 0.5x antibiotic-antimycotic at 37°C in 5% CO₂. Bradyzoites were grown for 3 days at 37°C in atmospheric CO₂ in RPMI medium (Invitrogen) buffered with 50 mM HEPES at pH 8.2 and supplemented with 5% dialyzed fetal bovine serum and 0.5x antibiotic-antimycotic. Bradyzoite media was changed daily in order to maintain the elevated pH.

Stable transgenic lines were generated by an established electroporation protocol using 20 μg of purified plasmid (Roos *et al.*, 1994). One day after electroporation, the media was exchanged to include 20 μg/ml mycophenolic acid and 50 μg/ml xanthine for selection of the HXGPRT cassette included in the plasmid. Clonal isolates were obtained by serially diluting transformed parasites into 96-well plates.

Dual luciferase assays requiring the transient expression of FF and RN luciferases in tachyzoites and bradyzoites were performed using 5 μg of FF plasmid (either pT5FF or pT0FF) and 5 μg of pT0RN. Four hours after electroporation, the media was changed to fresh tachyzoite or bradyzoite media and the parasites were grown for 24 h or 3 days,
respectively. A similar strategy was used to examine extracellular parasites expressing FF and RN luciferases. Three million freshly lysed tachyzoites were collected and electroporated with FF plasmid (either pT5FF or pT0FF) and pT0RN each at 1 μg plasmid / 10^6 parasites. After four hours, the media was exchanged and the parasites were allowed to grow for 24 hours before collection.

4.2.3 Fluorescent microscopy and relative GFP quantification

Stable transgenic P53L1-S1 or PT3L1-S1 (Table 1) parasites were grown for 24 h under tachyzoite conditions and for 5 days under bradyzoite conditions on confluent coverslips. Following fixation in 3% paraformaldehyde, the coverslips were stained in 10 μM Hoechst, rinsed in PBS, and mounted onto slides. Relative GFP quantification was performed as previously described using the Northern Eclipse software (Image Scanning Technology, Ontario, Canada) on a Leica DMIRB inverted microscope (Holmes et al., 2014). Briefly, at least 100 vacuoles per life stage per clonal line (total N ≥ 300) were analysed by densitometry. Relative bradyzoite fluorescence was expressed as a function of tachyzoite values, which were normalized to 100%. To assess the statistical significance of any bradyzoite-specific decrease in GFP expression, a one-tailed z test assuming unequal variances was performed using the Data Analysis pack available within Microsoft Excel 2007.

4.2.4 Generation of in vitro transcribed RNA fragments

DNA fragments incorporating the T7 RNA polymerase promoter were generated by PCR using plasmid templates (see Appendix B for appropriate template and oligonucleotide combinations). Reactions were performed according to the manufacturer’s
instructions (OneTaq; NEB). PCR fragments were cleaned by phenol:chloroform extraction, ethanol precipitated, and resuspended in DEPC-treated water prior to use for in vitro transcription.

RNA fragments were generated in a standard T7 RNA polymerase reaction mixture consisting of 50 mM Tris pH8.0, 10 mM MgCl₂, 2 mM spermidine, 25 mM NaCl, 2.5 mM rNTPs, 1 mM DTT, 1 U of pyrophosphatase and 20 U of RNase inhibitor (Thermo Scientific). Reactions were incubated at 37°C for 3 h after which point 1 U of DNase I (Promega) was added. The reaction mixture was incubated another 30 minutes to degrade the DNA template. Synthesized RNA was cleaned by phenol:chloroform extraction, ethanol precipitated, resuspended in DEPC-treated water and quantified on a NanoDrop spectrophotometer (Thermo Scientific).

4.2.5 Northern Blot

A DIG-labeled probe corresponding to a 273 bp region of the GFP coding sequence was generated by PCR using a plasmid template. The reaction was performed according to the manufacturer’s instructions (OneTaq; NEB) with the exception that the reaction mixture contained 100 μM dA/G/CTP, 67 μM dTTP, and 33 μM DIG-11-dUTP (Roche; Eisel et al., 2008). The reaction was run in conjunction with non-labeled dNTP mix and ran on an acrylamide gel to ensure a gel shift due to labeled nucleotide incorporation. The probe was phenol:chloroform extracted, ethanol precipitated, and resuspended in DEPC-treated water prior to use.

The northern blot protocol was adopted from the DIG Application Manual for Filter Hybridization from Roche (Eisel et al., 2008). One microgram of in vitro synthesized RNA
was run on a 1.5% formaldehyde-agarose gel and transferred overnight to a nylon membrane. After crosslinking, the blot was pre-warmed at 50°C in 5 ml of hybridization buffer (Roche) and hybridized overnight at 50 °C with 2 μl of snap-cooled DIG-labeled probe. The blot was then washed twice in 2X SSC/0.1% SDS (1X SSC: 150 mM NaCl, 15 mM sodium citrate) for 5 minutes at room temperature and twice in 0.1X SSC/0.1%SDS for 15 minutes at 65°C. Blocking, incubation with α-DIG-alkaline phosphatase (1:10 000), and exposure were performed according to manufacturer’s instructions (Roche).

4.2.6 S1 aptamer affinity purification

RNA segments containing the S1 aptamer were isolated using a protocol adapted from Srisawat and Engelke (2001). Fifty micrograms of S1-containing in vitro synthesized RNA were briefly heated to 75°C and snap-cooled on ice before addition to 25 μl of streptavidin-agarose beads (Thermo Scientific) in the presence of 200 μl of binding buffer (50 mM HEPES pH 7.4, 10 mM MgCl₂, 100 mM NaCl, 1 mM DTT, 0.1% Triton X-100, 10% glycerol, 20 mM vanadyl ribonucleoside complexes; modified from Srisawat and Engelke, 2001). The RNA was allowed to incubate while rotating for 1 h at 4°C. The agarose resin was pelleted at 1000 rpm for 2 minutes at 4°C on a tabletop centrifuge and washed in 200 μl binding buffer for 5 minutes a total of ten times. The beads were eluted in binding buffer supplemented with 5 mM D-biotin (BioBasic) while rotating at 4°C for 30 minutes. The supernatants from each sample were ethanol precipitated in the presence of 5 μg of linear polyacrylamide carrier and resuspended in DEPC-treated water. Samples were then reverse transcribed using a GFP-specific primer according to the manufacturer’s instructions (M-MLV; BioBasic) with the exception of supplementing the reaction mixture with dNTPs to final concentration of 2 mM. The cDNA was PCR-amplified to generate a
324 bp product corresponding to a segment of the *GFP* coding sequence. See Appendix B for relevant oligonucleotides.

### 4.2.7 Affinity purification of bacterially expressed CP-GFP-SBP

The pET_CP-GFP-SBP vector was transformed into BL21 competent cells and grown overnight in a 2 ml starter culture. A one litre culture was grown to 0.5 OD$_{600}$ and induced with 0.2 mM IPTG at room temperature overnight. The bacteria was isolated by centrifugation and the pellet lysed by sonication in denaturing lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 0.1% Triton X-100, 20 mM imidazole, 8 M urea) because it had been determined by SDS-PAGE that CP-GFP-SBP was restricted to inclusion bodies. The supernatant was transferred to a Ni-NTA column previously equilibrated in five volumes of denaturing lysis buffer. Following two washes with denaturing lysis buffer, the CP-GFP-SBP protein was allowed to refold on the column using four washes with non-denaturing wash buffer (same recipe omitting urea). CP-GFP-SBP was then eluted in non-denaturing wash buffer supplemented with 250 mM imidazole and analysed by SDS-PAGE for purity.

### 4.2.8 Affinity purification of MS2 aptamer from tachyzoite lysates

This procedure was performed with the intention of selectively purifying a transcript containing the *LDH1* 5′UTR and the MS2 aptamer from tachyzoite lysate using purified, bacterially expressed CP-GFP-SBP. Generally, the methodology described below was adapted from Slobodin and Gerst (2010; 2011) who optimized a system for the recovery of MS2 aptamer-containing transcripts from mammalian lysate using an inducibly expressed CP-GFP-SBP. One important modification from the aforementioned method is the use of
a smaller MS2 aptamer, containing two MS2 loop repeats (Said et al., 2009) instead of twenty four (Slobodin and Gerst, 2010). Furthermore, buffer compositions were altered from Slobodin and Gerst (2010) to milder, less stringent buffers to promote MS2 aptamer binding to recombinantly expressed CP after formaldehyde crosslinking and cell lysis (Said et al., 2009; Corcoran et al., 2012).

4.2.8.1 Preparation of tachyzoite lysates

Extracellular PT5L1-MS2 tachyzoites were collected from seven 10 cm culture dishes and pelleted at 2500 rpm for 10 minutes at 4°C. After being washed one time in PBS, the cell pellet, consisting of approximately $10^8$ parasites, was resuspended in isotonic crosslinking buffer (20 mM HEPES-KOH pH 7.4, 150 mM KCl, 1.5 mM MgCl$_2$, 1 mM DTT, 0.1% formaldehyde) and incubated on ice for 10 minutes to crosslink mRNA-protein complexes. Crosslinking was stopped through the addition of glycine to a final concentration of 250 mM and incubation on ice for 10 minutes. The cells were then pelleted at 6000 rpm for 5 minutes at 4°C on a tabletop centrifuge. After rinsing in isotonic wash buffer (IWB; isotonic lysis buffer without formaldehyde) the cell pellet was resuspended in 500 μl hypotonic lysis buffer (20 mM HEPES-KOH pH 7.4, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM DTT, 0.1% Triton X-100, 20 U/mL RNase inhibitor, 1 μl/ml Pefabloc protease inhibitor) and lysed through multiple freeze-thaw cycles followed by three short bursts of sonication on ice. The supernatant was collected and protein concentration was determined using the RC-DC protein quantification kit (Bio-Rad). Egg white avidin (BioBasic) was added at 5 μg/mg of lysate and the sample was incubated at 4°C while rotating for 1 h to block endogenous biotinylated proteins.
4.2.8.2 Preparation of CP-GFP-SBP-bound streptavidin-agarose beads

All steps were performed at 4°C and all wash and incubation steps were performed while rotating. Fifty microliters of streptavidin-agarose resin was equilibrated in 500 μl of IWB and pelleted at 1000 rpm for 2 minutes on a tabletop centrifuge. The beads were then concurrently bound by 5 μg CP-GFP-SBP and blocked for 90 minutes in isotonic blocking buffer (IWB supplemented with 2% BSA, 100 μg/ml yeast tRNA, 20 U/ml RNase inhibitor). To remove excess CP-GFP-SBP and blocking components, the resin was washed with five 5 minute washes in 500 μl IWB.

4.2.8.3 Affinity purification and western blot analysis

Five milligrams of prepared lysate was incubated with prepared agarose beads in IWB overnight while rotating at 4°C. The beads were then subjected to five or six 5 minute washes in 1 ml of IWB. The resin was eluted in 80 μl isotonic elution buffer (IWB supplemented with 6 mM D-biotin (BioBasic) for 30 minutes while rotating at 4°C. The formaldehyde crosslinking was then reversed by addition of an equal amount of 2X crosslink reversal buffer (100 mM Tris pH 7.0, 10 mM EDTA, 20 mM DTT, 2% SDS) and heated at 65°C for 1 h. Twenty microlitres of the sample was then run on a 10% SDS-PAGE acrylamide gel which was then processed by silver staining or western blot. Silver staining was performed using an established procedure (Shevchenko et al., 1996). After transferring to a nitrocellulose membrane and blocking in 5% milk, the blot was probed with α-GFP (1:2500; Rockland) or α-human PABPC3 (1:1000; Applied Biological Materials). The blot was then incubated in horseradish peroxidase-conjugated α-goat (1:10 000) or α-rabbit (1:20 000) secondary antibodies as appropriate and chemiluminescent signals were visualized on an Alpha-Imager system.
4.2.9 Poly(A) tail length analysis

The length of the poly(A) tail of selected transcripts was estimated through a two-step RT-PCR approach (Murray and Schoenberg, 2008). Total RNA was isolated from intracellular tachyzoites or bradyzoites using guanidinium isothiocyanate reagent according to the manufacturer’s instructions (BioBasic), resuspended in DEPC-treated water and 20 mM vanadyl ribonucleoside complexes, and treated with 1 U DNase for 30 minutes at 37°C. A reverse transcriptase reaction was performed as previously described (Holmes et al., 2014) using an oligonucleotide designed to bind the poly(A) tail with high enough annealing temperature to function in subsequent PCR analysis (Murray and Schoenberg, 2008; Appendix B). PCR was performed using a standard reaction mixture except only a gene-specific forward primer was added at 0.2 μM. The sequence of the LDH1 and GRA1 forward primers were designed to anneal 124 nucleotides upstream of the poly(A) tail as determined by other studies (Appendix B; Yang and Parmley, 1997; Cesbron-Delauw et al., 1989). The PCR reactions were run on a 10% non-denaturing acrylamide gel at 200V for 1 h and stained with ethidium bromide prior to visualization using the Alpha-Imager system.

4.2.10 Dual luciferase assay

4.2.10.1 Stage differentiation trials

After the appropriate growth period, tachyzoite- and bradyzoite-infected fibroblast monolayers plates were rinsed briefly with PBS. The monolayers were then scraped and lysed in 200 μl passive lysis buffer (Promega). After a 15 minute incubation at room temperature, the lysate was centrifuged for 1 minute at 12 000 rpm on a tabletop centrifuge and the supernatant was transferred to a new tube. To obtain FF luciferase readings, 20 μl
of lysate was added to 100 μl FF luciferase buffer (20 mM Tricine, 10 mM MgSO₄, 5 mM DTT, 250 μM Coenzyme A, 250 μM ATP, 200 μM D-luciferin) as previously described (Crater et al., 2012). RN readings were obtained as previously described (Crater et al., 2012) using 20 μl of lysate and 100 μl RN luciferase buffer (100 mM K₂HPO₄/KH₂PO₄ pH 7.4, 500 mM NaCl, 1 mM EDTA, 0.02% BSA, 10 μM coelentraxine; Liu et al., 1997). All readings were performed with a 5 second integration time on a 20/20ª luminometer (Turner BioSystems). FF readings were normalized against the RN signal and bradyzoite relative fluorescent units (RFU)s were expressed as a function of tachyzoite RFUs. A one-tailed Student t test assuming unequal variances was performed using the Data Analysis pack available through Microsoft Excel 2007.

4.2.10.2 Extracellular dual luciferase assay

After growing for 24 h under optimal tachyzoite conditions, infected monolayers were scraped and the parasites were released from the lifted host cells by passing them twice through a 26 gauge needle. After centrifugation, the parasites were resuspended in high potassium buffer (25 mM HEPES-KOH pH 7.4, 142 mM KCl, 5 mM NaCl, 5 mM MgCl₂, 2 mM EGTA, 1 mg/ml BSA), which has been shown to cause stress granule formation in extracellular parasites (Lirussi and Matrajt, 2011). The parasites were then separated into six equal aliquots and assayed over a time course of three hours for their FF and RN luciferase activities as previously described (Crater et al., 2012) with the exception that BSA was omitted from the RN luciferase detection buffer.
4.3 Results and discussion

4.3.1 Adaptation of the S1 aptamer system for affinity purification

The *LDH1* 5′UTR has previously been shown to be responsible for conferring sustained translational repression to multiple transcripts when placed in a heterologous context (Holmes *et al.*, 2014). Since this activity depended on the presence of small structural element, it was hypothesized that an interaction was occurring between this *cis*-acting element and an unidentified *trans*-acting factor (Holmes *et al.*, 2014). Previous reports have suggested four general strategies for the identification of RNA-protein interactions. In the first method, an RNA-binding protein (RBP) likely to be involved in the interaction is immunoprecipitated and the association of the RNA in question is validated through detection techniques such as RT-PCR (Niranjanakumari *et al.*, 2002). While this method has the advantage of retrieving *in vivo* RBP-RNA interactions, it assumes that the RBP is known and only requires validation as an interacting partner, which is not the case for the *LDH1* 5′UTR. A second, more RNA-centric method relies on using a biotinylated version of the *cis*-acting element as a bait to selectively purify any *trans*-acting factors from cell lysate (Rouault *et al.*, 1989). While this method has the advantage of using a specific sequence to capture RBPs, it can be prone to non-specific binding from biotinylated moieties or other RBPs and relies on a non-specific elution (Rouault *et al.*, 1989; Slobodin and Gerst, 2011). Furthermore, this method presents a major technical challenge in the particular case of *Toxoplasma* since it is co-cultured with human fibroblasts and the potential of contaminating human protein in parasite lysates could obscure a genuine *Toxoplasma* RBP-RNA interaction. Because of this consideration, an option that preserves mRNP composition prior to cell lysis was required. This could be achieved by a
third option which uses a tagged oligonucleotide to capture the transcript in question through base pairing interactions (Baltz et al., 2012). While decent specificity can be achieved with this method, the complementary binding of target and bait would not occur if the selected region were obscured by RBPs or if it were already involved in double stranded interactions. The final option considered for retrieval of the LDH1-interacting trans-acting factor was an affinity purification that relied on the use of an RNA aptamer (Srisawat and Engelke, 2001; Slobodin and Gerst, 2010). The procedure requires the aptamer to be encoded within the transcript which could then be used to for retrieval of the transcript and its protein complement through the aptamer’s affinity for an anchored bait molecule. This system can achieve high target specificity similar to antibody-antigen interactions and also is amenable to competitive elution of target molecules which facilitates downstream analysis (Srisawat and Engelke, 2001, Slobodin and Gerst 2010; Slobodin and Gerst, 2011).

Because of these benefits, the S1 aptamer system was selected. This 44 nucleotide aptamer, which has been shown to selectively bind streptavidin with a dissociation constant of 7.0 x 10^{-8} M (Srisawat and Engelke, 2001), was engineered into a previously established reporter system directly after the GFP stop codon (Holmes et al., 2014; Figure 4.1A). To ensure that the inclusion of the aptamer did not affect stage specific translational repression, tachyzoites and bradyzoites expressing GFP reporters flanked upstream by either the LDH1 5’UTR or the non-regulated TUB8 5’UTRs were examined for their relative fluorescence (Figure 4.1B). While the GFP reporter flanked upstream by LDH1 displayed a 51% ± 6% (P = 0.999, P53L1-S1) bradyzoite-specific decrease in GFP fluorescence, PT3L1-S1 remained unregulated in the bradyzoite stage (Figure 4.1B). This is consistent with
previously reported results (Holmes et al., 2014) and indicates that the insertion of sequences directly downstream of the GFP stop codon do not alter the ability of the LDH1 5′UTR to confer sustained translational repression to transcripts in bradyzoites.

To assess whether the S1 aptamer could be used for affinity purification, an in vitro synthesized RNA fragment consisting of the LDH1 5′UTR, the GFP coding sequence, the S1 aptamer and the SAG1 3′UTR (5L1-GFP-5L1-3SAG1; Figure 4.1C upper panel) was made and incubated with streptavidin coated beads. After washes, the beads were competitively eluted using biotin, which binds streptavidin with a dissociation constant of 10^{-14} M and can completely dissociate the aptamer from the beads (Srisawat and Engelke, 2001). An RT-PCR was performed on the eluted fractions and the RNA fragment was not retrieved (Figure 4.1D, upper panel). Since large amounts of the RNA transcript were added to the initial binding step, the failure to retrieve it upon elution suggested that the S1 aptamer could not bind the streptavidin beads.

The S1 aptamer was engineered using an in vitro selection process and notably, it is one member of a family that was generated to selectively bind streptavidin (Srisawat and Engelke, 2001). While this aptamer family shared some sequence similarity, all members produced an identical secondary structure (Srisawat and Engelke, 2001; Figure 4.1A). Furthermore, the authors suggested that the S1 aptamer must adapt the conserved secondary structure for it to maintain its activity. It was therefore hypothesized that the S1 aptamer was unable to adopt the proper conformation in the context that it was used here. Using the MFOLD algorithm (Zuker, 2003), it was found the sequence of the S1 aptamer is predicted to extensively interact with the SAG1 3′UTR (Figure 4.1C, upper panel). To
assess if this predicted base pairing interaction was responsible for precluding the proper S1 conformation, an RNA fragment was generated as before while lacking the SAG1 3’UTR (Figure 4.1C, lower panel). This transcript, which expresses the S1 aptamer on its 3’ terminus, was predicted to allow the aptamer to fold upon itself (Figure 4.1C, lower panel) and was successfully bound and eluted from the streptavidin beads (Figure 4.1D, lower panel). This result underscores the reliance of the S1 aptamer on achieving a proper conformation for its functionality (Srisawat and Engelke, 2001).
Figure 4.1 The S1 aptamer does not affect regulation but must fold upon itself to bind streptavidin beads.

(A) Graphical representation of S1-containing GFP reporter plasmids. The S1 aptamer (Srisawat and Engelke, 2001) is placed directly after the GFP stop codon. Orange segments are derived from LDH1, green from GFP, purple represents non-regulated sequences. (B) Relative GFP fluorescence (RFU) of transformed parasites under tachyzoite and bradyzoite conditions. Bars show normalized average readings ± standard error ($N \geq 300$) obtained from at least three independent clones per construct. Dark bars, tachyzoites; light bars, bradyzoites; asterisk, bradyzoite-specific decrease where $P \leq 0.001$. (C) Predicted secondary structures of the S1 aptamer when placed in the context of the in vitro synthesized transcripts depicted in the inset. (D) RT-PCR of affinity purification samples. The washes were grouped in sets of two. L, ladder; FT, flow through; E1/E2 elutions; RT +/-, cDNA or NoRT control respectively.
Since the S1 aptamer appeared functional when placed in a 3’ terminal position, a transcript was engineered to express the HDV ribozyme directly downstream of the S1 aptamer (Figure 4.2A). Ribozymes are RNAs that possess enzymatic activity and this class self-cleaving viral ribozymes have been extensively used to produce transcripts with defined 3’ termination sites when appended directly downstream of the desired cleavage site (Figure 4.2A; Chowrira et al., 1994; Schürer et al., 2002). The HDV ribozyme, which is derived from the hepatitis delta virus genome, forms a conserved pseudoknot structure and causes the cleavage of transcripts directly at its 5’ terminus with minimal upstream sequence requirements (Figure 4.2B; Been, 2006; Chowrira et al., 1994). In contrast to other ribozymes that generate precise 3’ termini, the HDV ribozyme has a relatively low requirement for its Mg$^{2+}$ cofactor (1 mM), suggesting that it is ideally suited for use in biological systems, where the estimated amount of free Mg$^{2+}$ in the cell is similar to that needed for optimal HDV ribozyme activity (Chowrira et al., 1994). Importantly, the HDV ribozyme has been shown to be tolerant of certain sequence mutations as long as secondary structure is maintained. For example, nucleotide changes within the P1 stem cause little effect on cleavage efficiency as long as compensatory mutations are present to preserve Watson-Crick base paring (Been et al., 1992; Ananvoranich et al., 1998). Likewise, decreasing the P4 stem from the long wild-type sequence to a shortened stem with a terminal ultra-stable UUCG tetraloop shows little change in HDV cleavage activity (Figure 4.2B; Been et al., 1992; Mercure et al., 1998). The HDV ribozyme variant used in this study contains mutations in the P1 stem that maintain Watson-Crick base pairing and has shortened P4 stem with a UUCG tetraloop, which has been indicated to cause increased stability to the ribozyme (Mercure et al., 1998).
In order to assess if inclusion of the HDV ribozyme was able to produce 3’ terminal S1 aptamers in a transcript, an *in vitro* transcription reaction was performed to produce the full length 1426 nucleotide transcript which is composed of the *LDH1* 5’UTR, GFP coding sequence, S1 aptamer, HDV ribozyme and *SAG1* 3’UTR (Figure 4.2A). This full length transcript was anticipated to be processed by the HDV ribozyme in the presence of MgCl₂ to a 1027 nucleotide shortened version that included the *LDH1* 5’UTR, the *GFP* coding sequence and a terminal S1 aptamer (Figure 4.2A). For size comparison, these transcripts were produced along with a catalytically inactive version of the HDV ribozyme and the full length transcript without the S1 or HDV ribozyme sequences, consisting of 1296 nucleotides. The inactive ribozyme has a deletion of 1 nucleotide, G58, which removes a purine-purine pairing that has been shown to be essential for catalytic activity (Figure 4.2B; Been and Perrotta, 1995). A northern blot of the aforementioned transcripts was performed to assess HDV processing (Figure 4.2C). Large trails were detected under each of the samples, likely due to non-specific degradation products or through the accumulation of products arising from premature transcriptional termination of the T7 RNA polymerase. While a clearer image could have been obtained had the uppermost bands of each sample been gel extracted and resuspended in a MgCl₂-containing buffer to induce HDV ribozyme cleavage prior to running the blot, it clearly shows no specific accumulation of cleavage product in the presence of HDV ribozyme (Figure 4.2C, lane 3). This indicated that the HDV ribozyme was unable to produce a transcript with a 3’ terminal S1 aptamer in this context.

Similar to the S1 aptamer, the HDV ribozyme requires the formation of a specific secondary structure to achieve a functional state. Although these considerations were
noted, it is likely that the HDV ribozyme was unable to fold properly into its complex pseudoknot structure. It had been hypothesized that the modified P4 hairpin, containing the ultra-stable UUCG tetraloop, would help to drive the proper folding of the ribozyme since this has been reported to confer additional stability to the HDV ribozyme (Been et al., 1992; Mercure et al., 1998). Contrarily, previous reports have shown that sequences downstream of the HDV ribozyme can have great positive or negative effect on its functionality likely through promoting its correct folding or by creating unwanted base pairing with the ribozyme (Chowrira et al., 1994). Since the use of the S1 aptamer and S1-HDV ribozyme methodologies both fell victim to the similar drawback of RNA misfolding, it was abandoned in favour of a strategy that was less reliant on the adoption of complex RNA structures.
Figure 4.2 The HDV ribozyme does not produce S1 terminal transcripts.

(A) Graphical representation of S1-HDV \textit{in vitro} synthesized transcript and cleavage product. (B) Diagramme of properly folded HDV ribozyme used in this study. The stems of the pseudoknot and nucleotide G_{58} are annotated. (C) Northern blot of \textit{in vitro} synthesized transcripts. Lane numbering corresponds to the samples indicated under the blot.
4.3.2 Adaptation of the MS2 aptamer system for affinity purification

The MS2 loop aptamer consists of a small 19 nucleotide RNA hairpin that specifically binds the MS2 bacteriophage coat protein (CP) with dissociation constant of $3 \times 10^{-9}$ M (Said et al., 2009; Slobodin and Gerst, 2010). This interaction has been utilized for the development of affinity purification methodologies where a CP fusion protein binds a transcript encoding the MS2 loop aptamer and is then selectively withdrawn from protein lysate. For example, Slobodin and Gerst (2010) developed a CP fused to GFP and a streptavidin-binding peptide (SBP; Figure 4.3A). This CP-GFP-SBP was expressed and shown to co-localize with MS2 loop-containing transcripts in vivo, showing that the GFP moiety could be used for live cell imaging (Slobodin and Gerst, 2010). Furthermore the SBP portion was compatible with streptavidin-agarose resin and permitted a clean, competitive elution in the presence of biotin (Slobodin and Gerst, 2010). However, because the CP has a propensity to oligomerize in vivo, the authors suggested that it be should only be expressed while using an inducible system to prevent non-specific binding (Slobodin and Gerst, 2010). This system was adapted for use in Toxoplasma and the LDH1-GFP reporter system. However, because of the propensity for CP to oligomerize and because the MS2 aptamer was incorporated into a GFP cassette (Figure 4.3A), which would make live-cell transcript visualization impractical, it was decided to bacterially express the CP-GFP-SBP capturing molecule.

After incorporation into the pET vector system, a C-terminally histidine tagged CP-GFP-SBP was expressed in a bacterial system. As is common for GFP-containing proteins expressed in E. coli (Peternel et al., 2008), the recombinant protein partitioned to the insoluble fraction and was therefore solubilized in an 8 M urea buffer and allowed to refold
on the column. This procedure isolated the CP-GFP-SBP protein at high purity as assessed by SDS-PAGE analysis (Figure 4.3B, lane P). While the choice to bacterially express the CP-GFP-SBP had advantages over allowing it to be produced in vivo, it also raised several important technical issues. For example, while the cellular expression of CP-GFP-SBP has the advantage of being able to preserve the MS2-CP interaction along with the mRNP composition through formaldehyde crosslinking (Slobodin and Gerst, 2010; Slobodin and Gerst, 2011), using a bacterially expressed CP-GFP-SBP instead requires the interaction to occur post-fixation. Therefore, modifications were made to the recovery procedure that take this into consideration. For example, gentler lysis, binding, and wash buffers were modeled after those that were used for MS2 aptamer affinity purification using a bacterially expressed CP (Said et al., 2009; Corcoran et al., 2012).

After crosslinking in formaldehyde, approximately $10^8$ extracellular tachyzoites expressing the MS2-tagged GFP transcript were lysed in a gentle hypotonic lysis buffer, yielding a total of 5 mg total protein. Notably, this amount of total protein has been recommended as the minimum amount to use for this procedure as using input amounts significantly lower than this may not yield sufficient protein for downstream western blot analysis (Slobodin and Gerst, 2011). After blocking endogenous biotin moieties with avidin, the protein lysate was applied to a streptavidin-agarose resin that was pre-bound with CP-GFP-SBP. The resin was washed five or six times and eluted with biotin. When the samples from the affinity purification procedure were run on an SDS-PAGE gel, a single 48 kDa protein, likely corresponding to the CP-GFP-SBP targeting molecule, was observed in the eluate (Figure 4.3B). A silver staining procedure had been performed with the hopes that other protein bands would be seen and could be identified using a mass
spectrometry approach (Figure 4.3B). However, since only the CP-GFP-SBP band was observed, it was thought that perhaps any other isolated proteins were under the detection limit for silver staining. After repeating the affinity purification procedure either in the presence of absence of CP-GFP-SBP, a western blot was performed (Figure 4.3C). Probing with α-GFP revealed a 26 kDa band corresponding to the free GFP produced by the MS2-tagged transcript in the parasite lysate as well as a 48 kDa band in the eluted fraction corresponding to the CP-GFP-SBP fusion (Figure 4.3C). This indicates that successful binding and elution of the CP-GFP-SBP targeting molecule to the streptavidin-agarose resin was achieved through its SBP moiety. In order to assess if the MS2-transcript was able to be purified using this methodology, the western was probed for PABP with the thought that since it is a ubiquitous RBP, it should be retrieved upon successful affinity purification of any MS2-tagged transcript. Multiple Toxoplasma-reactive bands were seen in the parasite lysate when the blot was probed with α-human PABPC3 (Figure 4.3D). However, no PABPC3-reactive protein was observed in the eluted sample, consistent with the possibility that the CP-GFP-SBP targeting molecule was unable to capture the MS2-containing transcript (Figure 4.3D). In addition, after reversing the formaldehyde crosslinking from the same eluted sample, RT-PCR analysis was performed, however no products corresponding to the GFP amplicon were seen (data not shown). Although care was made to minimize the risk, technical issues such as post-lysis RNA degradation cannot be completely discounted. However, it is likely that the MS2-CP interaction did not occur.
Figure 4.3 CP-GFP-SBP binds streptavidin but not the MS2 aptamer.

(A) Cartoon representation of the materials used. Bacterially expressed CP-GFP-SBP fusion protein (green) should bind streptavidin-agarose resin (white) and the MS2 aptamer (pink). The MS2 aptamer is encoded directly after GFP in a reporter construct.

(B) Silver stained SDS-PAGE of affinity purification trial using PT5L1-MS2 parasites. Lanes: P, purified CP-GFP-SBP; L, protein ladder; FT, diluted flow through sample, W1-6, washes, E, biotin elution. (C) Western blot of affinity purification trial done in parallel with and without the CP-GFP-SBP targeting molecule. The blot was probed for GFP. Dark arrowhead outlines the 26 kDa GFP from the parasite lysate while the white arrow indicates the 48 kDa CP-GFP-SBP fusion. Lanes: as in (B); w/o, without CP-GFP-SBP; w/, with CP-GFP-SBP. (D) Western blot from (C) probed with anti-human PABPC3. Lanes are indicated as before; OE, over-exposed elution.
Two important issues could have disfavoured the binding of the MS2 loop aptamer by the CP. First, since the bacterially expressed CP-GFP-SBP was insoluble, it was solubilized and denatured through the use of 8 M urea. While the protein was subsequently allowed to refold, it is very possible that the CP was unable to adopt its proper conformation. This issue likely could have been avoided had the central GFP portion been removed from the CP-GFP-SBP fusion since GFP is known to aggregate into insoluble bacterial inclusion bodies (Peternel et al., 2008). Alternatively, a mutant CP that is defective in oligomerization exists (Said et al., 2009) and could have been used for expression of the fusion within Toxoplasma, with the hope that the MS2-CP interaction would have occurred under more natural conditions in the cell.

The second major issue that could have prevented MS2 binding echoes the problems encountered with the S1 aptamer approach, namely that the MS2 aptamer may not have folded into the necessary conformation to permit CP binding. The MS2 aptamer is a small, 19 nucleotide RNA hairpin that has a weak predicted free energy ($\Delta G = -6.80 \text{ kcal/mol}$; Said et al., 2009; Zuker, 2003). This indicates that its stability is relatively low and therefore an individual hairpin is prone to be influenced surrounding upstream and flanking sequences. To combat this issue, multiple units of the MS2 aptamer are typically expressed in tandem. For example, like this study, Said et al. (2009) expressed two MS2 hairpins in tandem while twenty four were used by Slobodin and Gerst (2010). By using such a large copy number of the MS2 hairpin (Slobodin and Gerst, 2010), it is likely that at least some
of the MS2 units could be shielded from the non-specific base pairing that would disrupt their activity.

In contrast, this study consistently used a minimalist approach when inserting sequences within the GFP reporter system with the goal of minimizing potential unintended consequences caused by the introduction of large sequence insertions. For example, instead of using the full length 84 nucleotide S1 aptamer (Srisawat and Engelke, 2001), the minimal sequence was used. Similarly, instead of opting to use a large copy number of MS2 hairpins, only two were used. While these minimal elements have been described as successful in purification procedures (Srisawat and Engelke, 2001; Said et al., 2009), they were not here. The primary difference between this study and others using minimal aptamer motifs is that in those cases the aptamers were rationally placed while accounting for the secondary structure of its surroundings. For example, in a proof of concept experiment using the S1 aptamer for the purification of RNaseP components, the S1 aptamer was placed at the end of a previously established stem loop (Srisawat and Engelke, 2001). This positioning likely encouraged the aptamer to form a self-folding domain since both upstream and downstream flanking sequences formed a stable, well defined RNA stem (Srisawat and Engelke, 2001).

In the future, such a strategy could be used for inserting an aptamer within the GFP reporter system and would likely yield more positive results. This could be accomplished in a number of ways. First, an aptamer could be inserted within a naturally occurring or an engineered stable hairpin, mimicking its previous use in RNase P (Srisawat and Engelke, 2001). Alternatively, multiple tandem copies of an aptamer could be used (Slobodin and
Gerst, 2010). However, to improve the folding efficiency of individual aptamers, these could be placed between two engineered long and stable RNA hairpins. Conceptually, these external hairpins would self-anneal and promote the internal sequences to act as a self-folding RNA domain.

4.3.3 The LDH1 poly(A) tail is not shortened during sustained translational repression

Controlling the length of a transcript’s poly(A) tail is a common mechanism for modulating its translational competency (Godwin et al., 2013). The poly(A) tail serves as a substrate for poly(A) binding protein (PABP) which in turn interacts with eIF4G, causing an increase in translation and circularization of the transcript in what has been termed the closed loop model of translation (Sonenberg and Hinnebusch, 2009). In contrast, a common theme amongst translationally repressed transcripts is their deadenylation which can either lead to their degradation or, if the poly(A) tail is kept very short, their sustained translational repression (Godwin et al., 2013). Since this mechanism is widely conserved and the LDH1 transcript is maintained in a translationally quiescent state in bradyzoites (Yang and Parmley, 1997; Holmes et al., 2014), changes in the polyadenylation state of the LDH1 transcript were examined in order to gain an insight into its stage-specific regulation.

An RT-PCR approach was undertaken to estimate the poly(A) tail length of LDH1 and, for comparative purposes, the constitutively expressed GRA1 transcript in intracellular tachyzoites and bradyzoites (Figure 4.4A; Murray and Schoenberg, 2008; Cesbron-Delauw et al., 1989). In this procedure, a modified oligo-dT primer is used as a substrate in a reverse transcriptase reaction for the synthesis of cDNA from polyadenylated mRNA (Figure 4.4A; Murray and Schoenberg, 2008). Next, a transcript-specific forward primer
is added and a conventional PCR is performed. The sizes of the resulting products represent a range spanning the length of the poly(A) tail (Murray and Schoenberg, 2008). Following this procedure, it was apparent that tachyzoites and bradyzoites produce an LDH1 transcript with similar poly(A) tail lengths (Figure 4.4B). After subtracting the 124 nucleotides resulting from the sequence upstream of the polyadenylation start site, the LDH1 poly(A) tail is estimated to vary between 25 and 200 nucleotides in both life stages. While it may seem as though a smaller poly(A) tail is favoured in both tachyzoites and bradyzoites due to its intensity on the gel (Figure 4.4B), it is important to note that PCR has a propensity for favouring the production of small amplicons over larger ones. Similar results were obtained for the non-regulated GRA1 in tachyzoites and bradyzoites (Figure 4.4B). However, the primary product appears significantly smaller than the predicted 124 bp minimal product based on the previous reporting of the polyadenylation site (Cesbron-Delauw et al., 1989), suggesting that the GRA1 3’UTR contains multiple polyadenylation sites.
Figure 4.4 The *LDH1* poly(A) is not shortened in bradyzoites.

(A) Cartoon representation showing the methodology of the poly(A) tail length estimation assay. Primer PAT-Rv binds within the poly(A) tail through an oligo-dT region and is used to synthesize cDNA. A PCR is then performed upon the addition of a gene-specific primer.  (B) Acrylamide gel showing the poly(A) tail lengths of *LDH1* and *GRA1* transcripts in tachyzoites and bradyzoites. The sizes of the ladder bands are indicated in bp on the side on the gel. Note: for size estimation, 124 bp corresponding to sequences upstream of the polyadenylation site must be subtracted.
While the finding that the LDH1 poly(A) tail is unregulated during sustained translational repression runs contrary to most other investigated systems, a similar result has been found in the apicomplexan parasite *Plasmodium* (Braks et al., 2008). Similar to LDH1, the transcripts of P25 and P28 are maintained in a translationally quiescent state in female gametocytes. Upon fertilization, these transcripts are released and translated. Interestingly, northern blot analysis of these transcripts revealed that their poly(A) is maintained in an elongated state while repressed (Braks et al., 2008). Together, these results suggest that, contrary to well-studied metazoans whose transcripts undergo deadenylation while silenced and awaiting developmental progression, apicomplexans are not regulated in this manner. Importantly, a BLAST search against the *Toxoplasma* genome using the *Xenopus* cytoplasmic polyadenylation binding element protein, which is commonly involved in poly(A) tail shortening (Godwin et al., 2013), revealed no hits suggesting that this common regulatory mechanism may be absent. Furthermore, the long poly(A) observed in repressed transcripts in apicomplexan parasites raises interesting questions regarding the involvement of PABP.

One of the most well studied proteins involved in stage-dependent translational repression in *Plasmodium* is the DEAD-box helicase DOZI, which is a homolog of yeast DHH1. DOZI is required for sustained translational repression of P25 and P28 in female gametocytes (Mair et al., 2006). A study examining DOZI-associated factors involved in translational repression identified a variety classic proteins involved in translational repression, including the *Plasmodium* homolog of BRUNO and a number of ALBA proteins as well as PABP, which was the protein identified with the highest abundance (Mair et al., 2010). While it could easily be expected that this association with
translationally repressed transcripts has little biological significance aside from the obvious poly(A) binding activity, the apicomplexan PABP has several features that could merit investigation.

When encountered with conditions of cellular stress, a generalized translational repression is induced while a small subset of transcripts, corresponding primarily to stress-responsive gene products, are preferentially translated (Joyce et al., 2013). Intriguingly, PABP is preferentially translated under these conditions along with bradyzoite-specific transcription factors and translational repressors such as the *Toxoplasma* homolog of PUF1 (Joyce et al., 2013). This is a particularly interesting finding considering that translation is stunted and most transcripts are thought to be rapidly degraded under these conditions (Joyce et al., 2013). One might instead expect that the large scale transcript degradation hypothesized to occur upon stressful insult would yield an abundant source of available PABP to serve any immediate needs under stress.

Also, PABP has been shown to interact with the *Toxoplasma* ABLA proteins, which form mRNA granules under stress and are involved in translational control (Gissot et al., 2013). Furthermore, polyadenylated mRNA has been found to aggregate into stress granules upon stressful insult (Lirussi and Matrajt, 2011). Presumably, PABP would be similarly located. Intriguingly, a chance BLAST search of *Toxoplasma* using the stress granule marker TIA1 protein as a query revealed PABP as the top hit with an expected value of $2 \times 10^{-25}$. In metazoans, TIA1 is regarded as an important protein involved in the aggregation of stress granules through its C-terminal prion-like domain (Anderson and Kedersha, 2008). Interestingly, unlike the metazoan PABPs, *Toxoplasma* PABP is
predicted to contain a nearly 200 amino acid prion-like domain near its C-terminus (Lancaster *et al.*, 2014). These PABP features along with the observation that repressed transcripts display long poly(A) tails may suggest that it acts as a stress granule aggregator under stressful conditions.

4.3.4 Transcript-specific translational repression is not an immediate event

The transition of tachyzoites to bradyzoites is known to occur in response to cellular stresses that cause the phosphorylation of eIF2α (Narasimhan *et al.*, 2008). This causes a generalized translational repression which is maintained throughout the bradyzoite life stage (Narasimhan *et al.*, 2008). Recently it has been shown using a GFP reporter system that the *LDH1* 5’UTR is responsible for conferring a sustained translational repression to its transcript through a cis-acting element and that if this element was missing or mutated, repression was relieved in bradyzoites (Holmes *et al.*, 2014). This indicates that in addition to the generalized translational repression mediated through eIF2α phosphorylation, transcript-specific mechanisms are also important for mediating long term translational control. While not mutually exclusive with the generalized model of translational repression, this raises the question as to whether the transcript-specific regulation of *LDH1* is an early event, occurring rapidly after stressful insult or a gradual long-term process. If it were to occur rapidly, it would be expected that *LDH1* would be repressed upon stressful insult relative to a transcript that remains unregulated in bradyzoites. In contrast, if transcript-specific repression were a gradual process, *LDH1* would show similar expression relative to a control transcript that remains constitutively expressed in bradyzoites. Determining the relative speed at which *LDH1* is repressed would allow for insight into transcript-specific translational repression in *Toxoplasma*. 
In order to answer this question, the previously described GFP reporter system was converted to a dual luciferase system. Notably, GFP is a very stable protein with an estimated half-life of 26 h (Kitsera et al., 2007), making it unsuitable for studying rapid changes in post-transcriptional gene regulation. In contrast, firefly (FF) and Renilla (RN) luciferases have half-lives of approximately 3 hours making them more ideal for the study of rapid changes in gene expression (Leclerc et al., 2000). Therefore, the GFP coding sequence was exchanged for the corresponding FF and RN sequences in order to create plasmids that would express FF in a regulated (PT5FF) and unregulated manner (PT0FF) while RN would be under the control of unregulated upstream and downstream sequences (PT0RN) according to previously established conventions (Figure 4.5A; Holmes et al., 2014). Importantly, when assessed for their relative expression in tachyzoites and bradyzoites, repression was restricted to PT5FF in bradyzoites, indicating that the exchange of GFP for FF does not affect regulation (Figure 4.5B).

In order to assess the rapidity with which LDH1 encounters transcript-specific repression, extracellular parasites were subjected to a high potassium buffer and their relative FF expression was assessed over a time course assay (Figure 4.5C). This buffer mimics the host’s cytoplasmic ionic composition and would typically be seen as the parasite leaves its parasitophorous vacuole upon egress (Lirrusi and Matrajt, 2011). Also, this buffer has previously been reported to cause a robust stress in the parasite, inducing eIF2α phosphorylation and the aggregation of mRNAs into stress granules (Lirussi and Matrajt, 2011; Gissot et al., 2013). Interestingly, both PT5FF and PT0FF showed a similar decay over a three hour time period (Figure 4.5C). When normalized against the PT0RN internal control, it becomes apparent that PT5FF and PT0FF are expressed at similar relative levels
relative to one another throughout the selected time points (Figure 4.5D). This indicates that transcripts containing the \textit{LDH1} 5’UTR are not preferentially repressed after initial stressful insult and in contrast suggests that a period of generalized translational repression is induced upon stressful insult. These observations are consistent with the model postulated by Joyce and colleagues whereby a stressful insult causes rapid eIF2α phosphorylation and a generalized translational repression that affects the wide majority of transcripts independently of repressive \textit{cis}-acting elements like that seen in \textit{LDH1} (Joyce \textit{et al.}, 2013; Holmes \textit{et al.}, 2014). Furthermore, these results clearly indicate that the translational control observed in extracellular tachyzoites is different than that seen in fully developed bradyzoites since PT0FF and PCTRL are regulated differently in the two stressful situations despite both being characterized as sustaining eIF2α phosphorylation (Figure 4.5B, Holmes \textit{et al.}, 2014; Narasimhan \textit{et al.}, 2008; Joyce \textit{et al.}, 2010).
Figure 4.5 The *LDH1* 5’UTR does not confer immediate translational repression in extracellular parasites.

(A) Cartoon representation of plasmids used for the dual luciferase assay. Purple segments represent *TUB8* or *SAG1* sequences; orange, the *LDH1* 5’UTR; yellow and blue FF and RN coding sequences, respectively. (B) Relative expression of FF luciferase normalized against RN levels in tachyzoites and bradyzoites. Bars represent mean ± standard deviation. Yellow bars, unregulated PT0FF; orange, PT5FF; dark bars, tachyzoites; light bars, bradyzoites; asterisk, bradyzoite-specific decrease where *P* ≤ 0.001. (C) FF luciferase shows a time-dependent decay. Data points ± standard deviation (*N* = 3) expressed as a function of the initial timepoint. These data points are not normalized against RN luciferase. Orange, PT5FF; yellow, PT0FF. (D) Dual luciferase assay with data from (C) normalized against internal PT0RN control. Bars indicated mean ± standard deviation (*N* = 3).
Interestingly, although all stressful stimuli tested to date seem to result in the same central response of eIF2α phosphorylation and translational repression, the biological outcomes to the parasite can be quite different depending on the circumstances. For example, exposure to the extracellular environment activates the GCN2-like TgIF2K-D kinase, causing eIF2α phosphorylation and a generalized translational repression at the step of translational initiation which in turn induces the aggregation of transcripts into stress granules (Konrad et al., 2011; Lirussi and Matrajt, 2011). Furthermore, both eIF2α phosphorylation and formation of stress granules are essential for sustained viability of extracellular parasites indicating that this can act as a coordinated survival mechanism (Konrad et al., 2011; Lirussi and Matrajt, 2011). In contrast, intracellular tachyzoites undergoing amino acid starvation see eIF2α phosphorylation mediated through TgIF2K-C (Konrad et al., 2013a). However this stimulus leads to the autophagic loss of the parasite’s unique mitochondrion and inevitable cell death (Ghosh et al., 2011). Finally, a collection of other stimuli that induce cytoplasmic or endoplasmic reticulum stress activate TgIF2K-B and -A respectively and cause the conversion to bradyzoites, which are marked by mitochondrial dysfunction and the production of stress-responsive transcripts (Narasimhan et al., 2008; Toursel et al., 2000; Behnke et al., 2008).

4.4 Conclusion

It is therefore apparent that while many different stressful stimuli can initiate translational repression, care should be taken when substituting one study model for another. This has a direct consequence on the question of whether or not to revisit an aptamer-based affinity purification approach for the identification of the putative trans-acting factor involved in the sustained translational repression of LDH1. While important
lessons have been learned regarding aptamer selection and potential placement within a transcript, the current technological limitations require a recommended minimum of 5 mg total protein lysate from which to begin such an experiment with any hopes of achieving an identification by western blot (Slobodin and Gerst, 2011). In this study, attaining that amount of protein required seven 10 cm culture plates yielding $10^8$ extracellular tachyzoites. Bradyzoites grow at several orders of magnitude less than tachyzoites and the purification procedures currently available are far less efficient for the former than the latter. It would therefore make it highly impractical and would be ill advised to follow a similar attempt in the future.

In contrast, the study of the fate of the $LDH1$ transcript in bradyzoites and extracellular tachyzoites has the potential to yield great insight into post-transcriptional gene regulation in *Toxoplasma*. Results shown here demonstrate that $LDH1$, and likely other transcripts, are not regulated in their poly(A) tail length while translationally repressed. Also, sustained translational repression of $LDH1$ is not an immediate event and likely occurs in bradyzoites specifically. This could be achieved if a translational repressor were to be produced or activated specifically in bradyzoites. Conceptually, this trans-acting factor would be responsible for binding and inducing, either directly or indirectly, the sustained translational quiescence of $LDH1$ and other transcripts.
4.5 Works Cited


gene regulation are critical for *Plasmodium* zygote development. PLoS Pathogens. 6(2): e1000767.


CHAPTER V – General discussion

5.1 The emerging role of securinine as an inducer of differentiation

As an important component of traditional Chinese medicines, securinine has long to know to possess biological activities and one of the most characterized is its action as an antagonist of the GABA receptor (Raj and Luczkiewicz, 2008). Because it is capable of acting on the central nervous system of the host, displays anti-cancer activity, and causes the conversion of tachyzoites into bradyzoites, it has been proposed that securinine shows potential as an anti-toxoplasmosis in cancer patients (Raj and Luczkiewicz, 2008; Rana et al., 2010; Holmes et al., 2011). However the mechanism of action remains undescribed in Toxoplasma. Since the publication of our results, research into the anti-cancer effects of securinine has continued and yielded some very interesting results regarding its activities and potential mechanism(s) of action.

It has been revealed that securinine causes the cell cycle arrest of leukemia, colon, and breast cancer cells in the G1 phase, reducing their growth and proliferation (Gupta et al., 2011; Xia et al., 2011; Li et al., 2014). Furthermore, securinine has been shown to be a potent inducer of monocytic differentiation in leukemia cells and this activity is independent of its GABA antagonistic activities but involves DNA damage (Gupta et al., 2011). Interestingly, bradyzoites are characterized by a cell cycle arrest in the G1 phase and the induction of differentiation (Radke et al., 2003). Furthermore, these activities occur in the same range (IC₅₀ = 10 μM) that securinine has been shown to inhibit Toxoplasma growth and induce differentiation to bradyzoites (Gupta et al., 2011; Holmes et al., 2011).
Investigations into the molecular targets of securinine have discovered a few novel possibilities for its mechanism of action. In *Plasmodium*, securinine has been found to bind and allosterically increase the activity of the essential enzyme dUTPase and decrease parasite replication (Vu et al., 2013). dUTPase catalyses the conversion of dUTP to dUMP, an essential step in the production of dTTP and has been described as a potential drug target for apicomplexan parasites (Quesada-Soriano et al., 2010). However, it is important to note that a decrease, not an increase, of dUTPase activity has been linked to inhibition of parasite growth (Quesada-Soriano et al., 2010). Although securinine has been shown to activate *Plasmodium* dUTPase and cause inhibition of parasite growth, no mechanistic relationship between the two activities has been suggested or investigated (Vu et al., 2013).

In contrast, one of the biological targets of securinine in mammalian cells may have interesting applications for future investigations into *Toxoplasma* differentiation. Securinine has been shown to bind and inhibit protein disulfide isomerase (PDI) in mammalian cells (Hoffstrom et al., 2010). PDI is an important chaperone protein involved in mediating the correct folding of proteins in the endoplasmic reticulum by ensuring the correct formation of disulfide bridges (Obukuro et al., 2013). Its inhibition either through nitrosylation or by securinine has been shown to cause an accumulation of unfolded proteins in the ER lumen (Obukuro et al., 2013). Notably, this accumulation is known to activate the PERK-like eIF2α kinase TgIF2K-A, causing a generalized translational repression and bradyzoite differentiation in *Toxoplasma* (Narasimhan et al., 2008). Furthermore, production of nitric oxide (NO), which nitrosylates and inactivates PDI, is known to be upregulated by macrophages during infection and be necessary for bradyzoite differentiation in *Toxoplasma* in vivo, suggesting that an unexplored link between PDI and differentiation
may exist (Obukuro et al., 2013; Bohne et al., 1994). It is therefore tempting to speculate that inactivation of PDI by securinine is conserved in *Toxoplasma*. While NO has been thought to cause bradyzoite differentiation through inhibition of mitochondrial enzymes (Bohne et al., 1994; Tomavo and Boothroyd, 1995), it may also have an unexplored role in PDI inactivation and ER stress induction in bradyzoites. If future investigations were to find securinine binds and inhibits *Toxoplasma* PDI, which maintains 55% sequence similarity with its human counterpart, it could be used to specifically probe the involvement of PDI in mediating bradyzoite differentiation.

5.2 A unifying model of transcript-specific translational repression and bradyzoite development

There is overwhelming evidence that sustained cellular stress causes the differentiation of tachyzoites to bradyzoites. Furthermore, a generalized translational repression plays a vital role in this process. Taken together, recent results in stress-induced translational repression combined with insights into transcript-specific translational repression can be combined into a cohesive model. This model integrates translational repression and bradyzoite differentiation with mRNP granule accumulation and selective transcript storage and maintenance within the context of developmental progression in *Toxoplasma*.

Upon stressful insult, such as oxidative stress, the accumulation of nitrogen reactive species or unfolded proteins in the lumen of the endoplasmic reticulum, one of the four encoded eIF2α kinases is activated in *Toxoplasma* (Narasimhan et al., 2008). eIF2α is phosphorylated, reducing the turnover of eIF2-GFP and availability of charged ternary
complex inhibiting translational initiation (Sullivan et al., 2004; Sonenberg and Hinnebusch, 2009). Translating ribosomes complete elongation but after termination, are not able to re-initiate due to the lack of available charged ternary complex (Anderson and Kedersha, 2008). Transcripts stalled in the translational initiation stage accumulate in the cytoplasm (Anderson and Kedersha, 2008). As in metazoans, these stalled transcripts would accumulate into cytoplasmic granules known as stress granules (Anderson and Kedersha, 2008). This has been observed in extracellular parasites but has only been indirectly observed in intracellular parasites using RNA-binding proteins (RBP)s as markers of aggregation (Lirussi and Matrajt, 2011; Gissot et al., 2013; Cherry and Ananvoranich, 2014). Since stressful insult causes the immediate translational repression of transcripts that would remain unregulated in bradyzoites (Figure 4.5C,D) it could be hypothesized that most transcripts are recruited to stress granules without regard for their selection, at least in the short term. This could be mediated through a ubiquitous RBP with aggregation-like properties. While in metazoans this function is performed by the prion-like TIA1 (Anderson and Kedersha, 2008), in Toxoplasma it may be mediated by PABP which is predicted to have a prion-like domain, is a classical marker of stress granules, and has been shown to be involved in translational repression complexes in Plasmodium (Section 4.3.3; Anderson and Kedersha, 2008; Mair et al., 2010; Gissot et al., 2013).

Stress granules, which have been described as loosely aggregated complexes that remain in equilibrium with the translational machinery, help to withdraw the stalled transcripts from the limited translational pool (Stoecklin and Kedersha, 2013; Yamasaki and Kedersha, 2008). This withdrawal is thought to allow the translational machinery to favour stress-responsive transcripts for translation (Yamasaki and Kedersha, 2008). At
least some of these transcripts contain specific cis-acting elements that aid them to be preferentially translated under stress (Joyce et al., 2013). These transcripts include PABP, the translational repressor PUF1, and a variety of bradyzoite-specific AP2 transcription factors (Joyce et al., 2013). It therefore follows that their production is important for the conversion of tachyzoites into bradyzoites.

Notably, tachyzoite to bradyzoite conversion is a lengthy process, dependent on cell cycle progression, and it is common to see parasites expressing both tachyzoite and bradyzoite-specific markers as their cell division slows (Radke et al., 2003). This indicates that the parasite goes through a transitional period, where a balance exists between “tachyzoite” and “bradyzoite” protein synthesis. This balance is likely shifted towards “bradyzoite” production through two complementary mechanisms. First, the stress-induced expression of PABP and PUF1 (Joyce et al., 2013), and perhaps other transcript-specific translational repressor proteins, would increase the stability of mRNP aggregation through their ability to bind cis-acting elements in specifically repressed transcripts, such as that of LDH1. This would decrease the amount of competition that these “tachyzoite” transcripts would be able to provide against stress-responsive “bradyzoite” transcripts. Transcript-specific translational repressors are known to be important for the long-term aggregation and translational inactivation of stage-specific transcripts and the inclusion of transcript-specific translational repressors in granules has been characterized as a late stage of mRNP aggregation, signaling a maturation of the stress granules (Anderson and Kedersha, 2008). This has been observed in apicomplexan parasites. For example in Plasmodium, PUF2 is essential for the long-term storage of transcripts that are transcriptionally silent in sporozoites and female gametocytes and has granular distribution
(Gomes-Santos et al., 2011; Miao et al., 2013). Furthermore, PUF1 and the translational regulator ALBA proteins show granular distribution in bradyzoites (Liu et al., 2014; Gissot et al., 2013).

In the second complementary mechanism, the production of stress-induced AP2 transcription factors initiates a cascade of downstream bradyzoite-specific transcription (Behnke et al., 2010; Behnke et al., 2008). This would increase the relative proportion of “bradyzoite” transcripts that could compete for the translational machinery against the disadvantaged “tachyzoite” transcripts that are assembled in weakly associated stress granules. Taken together these two mechanisms would form a positive feedback loop where the increase in “bradyzoite” transcripts leads to more “bradyzoite” proteins, presumably including more transcript-specific translational repressors. In turn, this increases the specificity and stability of mRNP aggregates, causing their maturation from loosely assembled stress granules into more stable germ-like granules, decreasing their ability to compete for translational machinery and causing more efficient bradyzoite protein production. Coupled with the inevitable degradation and removal of “tachyzoite” transcripts whose genomic loci are no longer transcribed, this mechanism would be consistent with a model of progressive bradyzoite differentiation. Interestingly, it has been shown that the expression of bradyzoite-induced transcripts shifts gradually over the period of days until reaching maturation, consistent with this hypothesis (Cleary et al., 2002).

Upon reaching a certain state bradyzoite maturation, the pressure on the translational machinery may decrease. Although eIF2α phosphorylation is maintained throughout the bradyzoite stage (Narasimhan et al., 2008; Konrad et al., 2013), transcripts that are
translationally quiescent during the initial stages of a stressful insult become translationally permissive in bradyzoites (Section 4.3.4). This suggests that as mRNP storage granules stabilize and the transcription of tachyzoite genes ceases, the subset of transcripts that are able to translated under the conditions of generalized translational repression expands somewhat. Again this is supported by the observation that the transcription of bradyzoite genes occurs gradually as the parasite matures (Cleary et al., 2002).

Finally, the process of bradyzoite to tachyzoite transition likely works in a manner similar to the above hypothesized mechanism of bradyzoite conversion. Similar to bradyzoite differentiation, the reactivation of bradyzoites to tachyzoites has been shown to be a gradual process, with the first signs of tachyzoite antigens being produced in approximately 18 hours after reactivation is induced (Radke et al., 2003). This would be consistent with a model whereby upon the relief of stress, eIF2α phosphorylation decreases, likely causing an uptick in translation (Konrad et al., 2013). Essential tachyzoite transcripts that were tightly stored in germ-like storage granules would gradually emerge from repression and enter the translational pool. The products of these stored transcripts would then drive metabolic and transcriptional changes, causing a cascade that amplifies the conversion from bradyzoite to tachyzoite. This hypothesis is consistent with previous research performed in Plasmodium where the premature release of transcripts from storage granules in salivary gland sporozoites, some of which are transcription factors, causes the premature progression into liver stage parasites (Gomes-Santos et al., 2011).
5.3 Potential components and characteristics of sustained translational repression

The above stated hypothesis supposes heavily on the existence of mRNA-containing germ-like granules in *Toxoplasma* bradyzoites. While these have not been directly observed to date, they are likely to exist since proteins involved in translational repression have displayed granular localization in bradyzoites (Liu *et al.*, 2014; Gissot *et al.*, 2013; Cherry and Ananvoranich, unpublished results). Furthermore these observations echo the composition of mRNA-containing germ-like granules found in the salivary gland sporozoite and female gametocyte stages of *Plasmodium* development (Gomes-Santos *et al.*, 2011; Mair *et al.*, 2010). It therefore follows that the components of germ-like granules in bradyzoites would be similar to what is found in their *Plasmodium* cousins.

Transcripts that undergo sustained translational repression are likely sequestered in germ-like granules. This would most assuredly include *LDH1*, and likely other transcripts that have been suggested to be post-transcriptionally silenced in bradyzoites such as the RNA-binding *TgDRE* and the canonical tachyzoite marker *SAG1* (Yang and Parmley, 1997; Dendouga *et al.*, 2002; Cleary *et al.*, 2005). Undoubtedly, other transcripts would be sequestered to these granules and would likely have the general function of being important for early tachyzoite survival; prime candidates would be tachyzoite-specific transcription factors, similar to what has been described in *Plasmodium* (Gomes-Santos *et al.*, 2011). Verification of transcripts recruited into germ-like granules could be accomplished by a fluorescent in situ hybridization approach.

Since transcripts require specific *cis*-acting elements for sustained translational repression in bradyzoites, translational repressor proteins that bind specific sequences are
also likely to be included in germ-like granules. Examples in *Toxoplasma* likely include PUF1 and ALBA proteins, all of which show granular distribution in bradyzoites (Liu *et al.*, 2014, Gissot *et al.*, 2013). Again this echoes findings in *Plasmodium* and metazoans (Mair *et al.*, 2010; Voronina *et al.*, 2007). These proteins would be responsible for the sustained translational repression of specific transcripts through the use of cis-acting elements. For example, *in vitro* assays show that *Toxoplasma* PUF1 binds the consensus PUF binding element from repressed *Plasmodium* transcripts (Liu *et al.*, 2014). This consensus bears a striking resemblance to the unpaired nucleotides within the small regulatory hairpin in the LDH1 5’UTR (Holmes *et al.*, 2014) and the verification of this interaction through *in vitro* binding assays and *in vivo* functional assays is an obvious progression of this work.

The final component of germ-like granules in bradyzoites would be proteins involved in the storage and maintenance of transcripts. Potential examples include the DEAD-box helicase homolog of *Plasmodium* DOZI and PABP. In *Toxoplasma*, this helicase displays granular distribution in bradyzoites and is capable of rescuing a temperature sensitive yeast DHH1 mutant indicating that it likely fulfills the same function in stress response (Cherry and Ananvoranich, unpublished results; Cherry and Ananvoranich, 2014). Furthermore, DOZI has been shown to be essential to the maintenance of germ-like granules in *Plasmodium* (Mair *et al.*, 2006). In contrast to metazoans, translationally repressed transcripts in *Toxoplasma* and *Plasmodium* maintain long poly(A) tails (Godwin *et al.*, 2013; Figure 4.4; Braks *et al.*, 2008), indicating that PABP is likely a major component of mature germ-like granules. PABP has indeed been found as a major component of DOZI-associated granules in *Plasmodium* and other potential protein candidates contained within
bradyzoite germ-like granules likely mirror those found within similar granules in *Plasmodium* (Mair *et al.*, 2010). Future studies, which could be facilitated with the novel application of CRISPR/Cas9 technology to *Toxoplasma* (Shen *et al.*, 2014; Sidik *et al.*, 2014), could be directed towards their localization and stress-related functions.

### 5.4 Conclusions and Significance

This work has focused on two areas, the first of which is the discovery of anti-*Toxoplasma* compounds. The serendipitous finding that securinine is an inducer of bradyzoite conversion may signal that it has applications in future studies regarding stress-response and differentiation. Furthermore, since it is a biologically active molecule that has been successfully used in animals and humans, it may be possible to use securinine, or a derivative thereof as a medicinal tool for treating or preventing recurrent toxoplasmosis. Notably, this work the first and only to show that securinine can induce cellular differentiation outside of metazoans.

The second area of research focuses on outlining the features of transcript-specific translational repression in bradyzoites. These studies show that transcripts are selected for long-term sustained translational repression through the use of *cis*-acting elements. This suggests the involvement of a yet to be identified *trans*-acting factor that specifically recognizes the transcript. This work is the first to functionally characterize a *cis*-acting element involved in post-transcriptional regulation in *Toxoplasma*.

This work also shows that differences exist between metazoan and apicomplexan sustained translational repression, namely through maintenance of the poly(A) tail. Finally, the observation that transcripts undergo a tight translational repression after initial stressful
insult that is later not as strict in mature bradyzoites suggests that the mechanisms regulating translational control change throughout bradyzoite development.
5.5 Works Cited


Appendix A. List of oligonucleotides used in Chapter 3

Primer combinations used to create each construct are listed. Inserted restriction sites are underlined. Red nucleotides represent mutated or inserted nucleotides.

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Appendix B. List of oligonucleotides used in Chapter 4

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<td>3UTRSAG1</td>
<td>GGATCCCCCTCGGGGGGGCAAGAAT</td>
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**RT-PCR**

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<th>Construct</th>
<th>5'UTR</th>
<th>3'UTR</th>
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<tr>
<td>T7-5L1-GFP-S1 /</td>
<td>GFP cDNA</td>
<td>GCCATGTGTAATCCAGC</td>
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<tr>
<td>T7-5L1-GFP-S1-3SAG1</td>
<td>GFP fragment</td>
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<td>3GFP1940</td>
<td>GTCGTGGCGCTTCATAG</td>
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**Northern probe**

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<td>pT5L1-DIG</td>
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<td>5GFP1667</td>
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**PAT assay**

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<th>PAT_Rv</th>
<th>cDNA</th>
<th>PAT_Rv</th>
<th>5'UTR</th>
<th>3'UTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH1-poly(A)_tail</td>
<td>PAT_Rv</td>
<td>GTG TGT GTG TGT GTC TCT TTT TTT TTT TTT T</td>
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<td></td>
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<tr>
<td>GRA1-poly(A)_tail</td>
<td>PAT_Rv</td>
<td>TTG TCC ACT GTC ATG TCG AAA AC GTC GTC GTG TGT GTC TCT TTT TTT TTT T</td>
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